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# THE EFFECT OF NaCl ON THE PHAGE-BACTERIUM REACTION\*

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(Accepted for publication, May 11, 1937)

Investigations dealing with the effects of salts on bacteriophage have been rather limited in scope and predominantly qualitative or roughly quantitative in character. For the most part efforts have been restricted to pointing out analogies between the balanced salt solutions required for the growth of common living organisms and those requisite for phage production. While the former data are essentially accurate and subject to adequate control the corresponding experiments with phage suffer from a lack of controlled experimental conditions and also from the use of inaccurate methods for determining phage quantitatively.

Consequently it is not surprising that there is no general agreement in the literature about salt effects. For example Brutsaert (1-2) concludes that phage may develop and bacterial lysis take place in peptone water without salt. Ciuca (3), Lisbonne and Carrere (4), and da Costa Cruz (5-7) on the contrary hold that electrolytes are essential for both phage production and bacterial lysis. Arloing and Chavanne (8) and Bordet and Renaux (9) stress not only the necessity for electrolytes in bacteriophagy but also the importance of the particular electrolyte used. The observations of Bordet (10), Stassano and de Beaufort (11), Planteureux (12), and Anciaux (13) indicate that the calcium ion is necessary for the lytic action of phage on bacteria.

Burnet and McKie (14) report experimental evidence for the protective action of divalent ions against inactivation of phage by heat and dyes. In Bronfenbrenner's work (15-16) it appears that the greater the concentration of electrolytes, the greater the percentage inactivation of phage by alcohol and acetone.

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Krueger and West (17) in an analysis of the kinetics of the phage-bacterium reaction in the presence of the manganous ion find that extremely small concentrations of  $Mn^{++}$  cause an acceleration of lysis due to a lowering of the lytic threshold, *i.e.*, the ratio of phage to bacteria requisite for lysis. There is a concomitant increase in the extracellular phage fraction and a decrease in the total quantity of phage produced.

Northrop (private communication) has noted that the presence of increased concentrations of sodium chloride during the period of reaction between phage and susceptible organisms leads to an augmented end titre of phage. We felt that a study of this reaction might furnish a better understanding of the way electrolytes effect bacteriophagy and might also be of aid in analyzing the mechanisms of phage production and bacterial lysis.

### Methods

1 Quantitative determinations of phage were routinely made by the activity method of Krueger (18)

*a Unit of Phage Defined*—The minimum quantity which will cause complete lysis when added to a total of  $12.5 \times 10^7$  cells of *S. aureus* (a 16 hour culture grown on agar and counted by the centrifuged sediment method of Krueger (19)), in a total volume of 5 ml of beef infusion broth held at 36°C at pH 7.4, in infinite time.  $1 \times 10^{-10}$  ml of the standard phage prepared by adding 1 ml of phage to  $2 \times 10^9$  staphylococci contained in a total volume of 100 ml of broth and allowing the mixture to lyse, suffices to produce lysis under these conditions and therefore contained  $1 \times 10^{10}$  units of phage per ml.

*b* Since there is a linear relation between the time of lysis and the log (initial concentration of phage), *i.e.*  $\log [P]_0$ , an unknown may be run according to the following procedure

$[P]$ , concentration of phage in units per ml

$[P]_0$ , initial concentration of phage in units per ml

$[B]$ , concentration of susceptible staphylococci per ml

$[B]_0$ , initial concentration of susceptible staphylococci per ml

4 ml aliquots of appropriate dilutions in broth of phage-containing solutions are pipetted into standard tubes. To each is added 1 ml of a broth suspension of staphylococci prepared as described above and containing  $12.5 \times 10^7$  organisms per ml. The tubes are placed in the water bath shaker and after 1.5 hours readings are made first at 0.2 hour and later, as lysis is initiated, at 0.1 hour intervals until the arbitrary end point of  $8 \times 10^7$  cells per ml is reached. With this series

dilutions of the original standard phage are run in the same manner. Usually four dilutions  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  are used. Log  $[P]$  is plotted against the time of lysis of these standard dilutions. Knowing the time of lysis for a given dilution of the unknown sample, the original concentration of phage in the mixture may be determined by reference to the standard curve.

2. The bacterial suspensions used in all the experiments described consisted of freshly harvested 16 hour cultures of *Staphylococcus aureus* (strain S<sub>3</sub>K) grown on agar and washed once in Locke's solution. Broth was standard beef infusion containing 1 per cent Difco Neopeptone, 0.5 per cent sodium chloride, and adjusted to pH 7.4.

3. Except where otherwise indicated, a volume of 1 M NaCl sufficient to give a final concentration of 0.25 M NaCl was used in the entire test series. These aliquots of one molal sodium chloride were added to beef infusion broth. In all cases control mixtures were included, consisting of the same constituents, similarly treated except that the volume of the stock solution of 1 M NaCl used was replaced by an equal volume of physiological saline 0.85 per cent (0.145 M).

4. *Growth Curves*—To 38.5 ml of broth and 17.5 ml of 1 M NaCl, 14 ml of a broth suspension of staphylococci containing  $12.5 \times 10^7$  bacteria per ml were added. This mixture and a control mixture were placed in the water bath shaker at 37°C. Turbidity readings were made directly or on an aliquot sufficiently diluted to come within the range of a formalinized standard series (range  $5 \times 10^7$  to  $20 \times 10^7$  bacteria per ml). Readings were made at intervals of 0.5 hour over a period of 4 hours, diluting the preparations when necessary to keep  $[B]$  within the range of the turbidity standards.

5. *Rate of Phage Production and Phage Distribution As Related to Bacterial Growth*—To 31.5 ml of broth were added 7 ml of standard staphylococcus phage diluted 1/1000 (final concentration of phage =  $1 \times 10^8$  units per ml), 14 ml of staphylococcus culture containing  $12.5 \times 10^7$  bacteria per ml, and 17.5 ml of 1 M NaCl. This was paralleled by a control mixture. These suspensions were placed in the water bath shaker at 37°C. At intervals of 0.5 hour turbidity measurements for bacterial growth were made on 5.0 ml aliquots. At the same time 1 ml was removed from the mixture, diluted 1/10 and kept in ice and salt for subsequent titration with the remainder of the series. A 5 ml aliquot was transferred to a 15 ml centrifuge tube, iced, and centrifuged for 20 minutes at 2100 R.P.M. 1 ml of the supernatant fluid from the centrifuged sample was diluted with 9.0 ml broth to be titrated for the amount of extracellular phage. The quantity of intracellular phage was then determined by difference: total phage - extracellular phage = intracellular phage.

6. *Control to Determine the Effect of NaCl on Phage Alone*—Phage left in infusion broth with 0.25 M NaCl at 37°C for 4 hours and at 0 for 24 hours was subsequently titrated.

7. *Control to Determine the Effect of NaCl on Staphylococci Alone*—A suspension of staphylococci,  $12.5 \times 10^7$ /ml, was left in the NaCl 0.25 M infusion broth for 3



hours at 0°C and the suspension used in a titration of standard phage along with a control series. A similar suspension, initially containing  $2.5 \times 10^7$  B/ml was grown in 0.25 M NaCl for 3.5 hours, diluted to  $12.5 \times 10^7$  B/ml, and used in a titration of standard phage along with a control suspension.

8 For determining the salt effect at the lytic threshold in static mixtures, aliquots of 5 ml total volume containing  $1 \times 10^8$  B/ml and varying concentrations of phage, were left at 0°C for 0.75 hour and then were placed in the water bath shaker at 37°C. The ratios of  $\log [P]_0/[B]$ , represented in these mixtures ranged from 1.0 to 2.0. Turbidity measurements were made on these mixtures at 0.10 hour intervals.

9 In order to determine the relative effect of salt on bacteria and on phage in mixtures at the lytic threshold, 9 ml of broth, 2 ml of a  $10^{-4}$  dilution of standard phage, 5 ml of 1 M NaCl solution, and 4 ml of a suspension of staphylococci containing  $12.5 \times 10^7$  organisms per ml were mixed and placed in the water bath shaker at 37°C. A similar mixture without the salt was set up as control. At 0.4 hour before the end-point of lysis (time determined previously in an identical mixture) two 5 ml aliquots of the control mixture and two 5 ml aliquots of the test mixture were removed from the bath, placed in 15 ml centrifuge tubes, the tubes packed in ice and salt in centrifuge cups, and centrifuged in the cold room for 12 minutes. The supernatant fluids of one test portion and one control were interchanged and the organisms uniformly resuspended. Two portions as controls, one with 0.25 M NaCl and one without, were resuspended without interchanging supernatants, and were returned in standard size tubes to the bath at 37°C. The end-point of lysis for these four suspensions was observed, along with that of the two control tubes which were left in the bath at 37°C during the treatment of the other four portions. The lysates of these test portions were subsequently diluted and titrated in the usual manner for total phage.

10 To determine the possible relative osmotic effect of the salt of a divalent anion, mixtures of phage and bacteria were set up in broth containing various concentrations of NaCl and  $\text{Na}_2\text{SO}_4$ , and the times of lysis noted. The lysates were also titrated for  $[P]$ .

11 The rate of oxygen consumption of the *Staphylococcus aureus* strain was measured during normal growth, during growth with phage present, and during growth with 0.25 M NaCl and phage present. The constant volume type of manometric apparatus was used with flasks of approximately 20 ml capacity. 1 ml of a staphylococcus suspension containing  $2 \times 10^8$  organisms per ml was added to 1 ml of beef infusion broth. Similar mixtures containing  $5 \times 10^6$  phage units in the 1 ml of broth or with 0.50 M NaCl in the 1 ml of broth-phage mixture were prepared. 0.4 ml of 20 per cent NaOH was added to the filter papers placed in the absorption chambers of the flasks. The mixtures were allowed to come to equilibrium in the 37°C water bath shaker (90-100 oscillations per minute) for a period of 0.5 hour before the stop-cocks were closed. Readings were made over a period of 2 hours at intervals of 10 minutes.

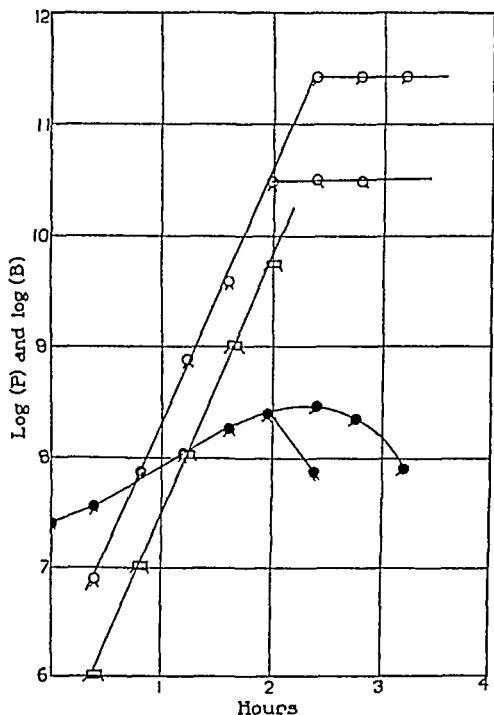


FIG 1 Composite plot of several experiments in which staphylococci were grown at  $36^{\circ}\text{C}$  in the presence of  $0.25\text{ M NaCl}$  and without  $\text{NaCl}$   $[B] = 2.5 \times 10^7$ ,  $[P] = 1.0 \times 10^6$  ○ = total  $[P]$  in the salt mixture □ = total  $[P]$  without salt, □ = extracellular  $[P]$  in the salt mixture □ = extracellular  $[P]$  without salt, ● =  $[B]$  in salt mixture ● =  $[B]$  in mixture without salt

## RESULTS

Kinetic analysis of the reaction between a susceptible staphylococcus and its homologous phage in the presence of  $0.25\text{ M NaCl}$  gave the following results

1 The growth curve of the staphylococcus strain remains normal under the influence of 0.25 M NaCl (Fig 1)

2 The activity of phage left in contact with 0.25 M NaCl at 0°C (18 hours) and at 37°C (4 hours) is not affected (Table I)

There is no effect on organisms exposed to salt action and then used for titrations of standard phage (Table II)

TABLE I

*Effect of Exposing Phage to 0.25 M NaCl at 0°C and 37°C*

$7.5 \times 10^9$  phage units/ml = initial phage concentration

NaCl in test solution 0.25 molal

Mixtures kept 18 hours at 0°C and 4 hours at 37°C

0°C		37°C	
Control	Salt	Control	Salt
Final [phage] $7.3 \times 10^9$	$7.5 \times 10^9$	$7.5 \times 10^9$	$7.4 \times 10^9$

TABLE II

*Effect of Exposing Organisms to 0.25 M NaCl and Subsequently Using Them for Titration of Standard Phage*

$12.5 \times 10^7$  B/ml left 4 hrs. at 0° in 0.25 M NaCl and in normal saline Both preparations subsequently used in titration of standard phage

Initial log [P] <sub>0</sub>	T <sub>lysis</sub> (hrs.)		End titre, log [P]	
	Salt	Control	Salt	Control
7.9	1.75	1.75	10.5	10.5
6.9	2.25	2.25	10.7	10.7
5.9	2.65	2.65	10.9	10.9

$2.5 \times 10^8$  B ml. grown at 37° in broth containing 0.25 M NaCl and in broth containing 0.145 M NaCl Both preparations subsequently used in titration of standard phage

Initial log [P] <sub>0</sub>	T <sub>lysis</sub> (hrs.)		End titre, log [P]	
	Salt	Control	Salt	Control
7.9	1.2	1.2	10.4	10.4
6.9	1.6	1.6	10.5	10.5
5.9	2.1	2.1	10.75	10.75

3 Sodium chloride, (0.25 M), has no effect on bacterial growth, rate of phage production, or phage distribution in growing mixtures of phage and bacteria up to the point of lysis. At this point, however, in the mixtures containing salt, lysis is delayed for approximately 0.6 to 0.75 hour as compared with the control mixtures. When the

end point of lysis is reached in the control mixture, the phage concentration rapidly increases in the salt mixture but no further bacterial growth takes place, an extraordinary phenomenon in view of all the experimental evidence establishing bacterial growth as an essential conditioning factor for phage production (15). The end titre of the lysate is five to ten times greater than that of the control mixture. Hence, at the point of lysis in the salt mixture the concentration of

TABLE III

*Determination of the Phage/Bacterium Ratios Requisite for Lysis without Bacterial Growth in the Presence and Absence of Salt*

NaCl 0.25 M (S)

Control (C)

[B] =  $1 \times 10^8$ /ml

Readings by turbidity, [B]  $\times 10^7$ /ml

Value of [P] / [B] initially	0.9		1.1		1.3		1.5		1.7		1.9	
Time	C	S	C	S	C	S	C	S	C	S	C	S
$t_{1/2}$												
0.2	10	10	10	10	10	10	10	10	10	10	10	10
0.4	12	12	12	12	12	12	12	12	10	10	10	10
0.6	16	16	16	16	16	16	12	12	6	9	6	9
0.7	18	18	18	16	12	16	7	12		9		9
0.8	22	22	16	16	8	12		12		6		6
0.9	12	20	5	12		10		6				
1.0	8	16		10		8						
1.1		14		8								
1.2		12										
1.3		10										
1.4		6										
Increase in end titre	3.6x		3.6x		3.0x		2.5x		3.0x		3.0x	
	(3 experiments)											

phage per bacterium, that is, the lytic threshold, is increased approximately five to tenfold, although the concentration of bacteria per milliliter is approximately the same as that of the control (Fig. 1)

4. Determinations of the lytic threshold in static mixtures with 0.25 M NaCl indicate that the lytic threshold, i.e. that mixture in which the ratio of phage to bacteria is such that lysis without growth takes place, is not changed. Although the time of lysis is delayed

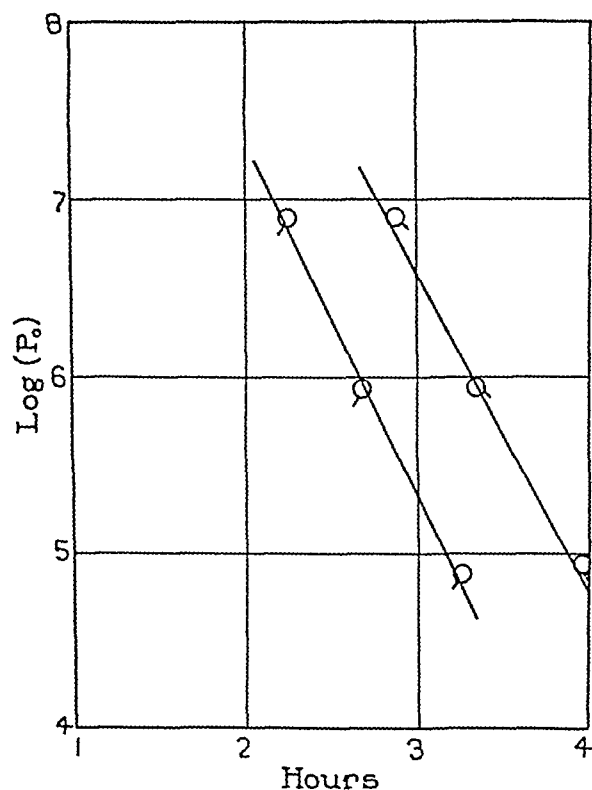


FIG. 2. The delay in  $t_{(1), (10)}$  caused by 0.25 M NaCl is independent of  $P_0$ . Q = phage and bacteria mixtures in broth containing 0.25 M NaCl,  $[B]_0 = 2.5 \times 10^7$ ,  $[P]_0 = 8.0 \times 10^6$ ,  $8.0 \times 10^5$ , and  $8.0 \times 10^4$  activity units/ml respectively. ○ = identical mixtures without salt. Temperature—36°C.

TABLE IV

*Effect of Varying Initial Concentration of Bacteria on Time of Lysis and Final Titre of Lysate, in the Presence and Absence of Salt*

[NaCl] = 0.25 M in test series

$[P]_0 = 1 \times 10^6$  units/ml

$[B]_0$		$2.5 \times 10^7$	$5 \times 10^7$	$7 \times 10^7$	$1.25 \times 10^8$
$T_{lysis}$	Control	2.6	2.68	2.78	3.15
	Salt	3.35	3.45	3.5	3.90
$\Delta T_{lysis}$	Control	0.75	0.77	0.72	0.65
	Salt	11.25	11.35	11.45	11.35
End titre	Control	12.0	12.0	12.1	12.0
	Salt	12.0	12.0	12.1	12.0

considerably in the presence of salt, there is no increase in bacterial growth but there is a slight increase in phage titre of the lysates, which is constant over the range of phage and bacterial concentrations used (Table III)

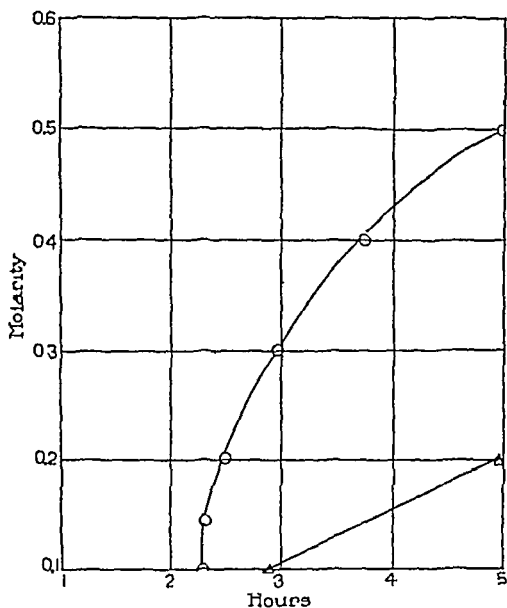


FIG 3 The effect of increasing  $[NaCl]$  on  $t_{(lys)}$   $[P] = 1 \times 10^6$  activity units/ml  $[B] = 2.5 \times 10^7$  B/ml with varying concentrations of NaCl (O), and Na<sub>2</sub>SO<sub>4</sub> (Δ) Increasing concentrations of both salts produce a considerable delay in the time of lysis

5 The delay of lysis by salt and the increase in end titre of the lysate are independent of the initial phage and bacterial concentrations used (Fig 2 and Table IV)

6 Within limits an increase in NaCl beyond 0.20 M causes a corre

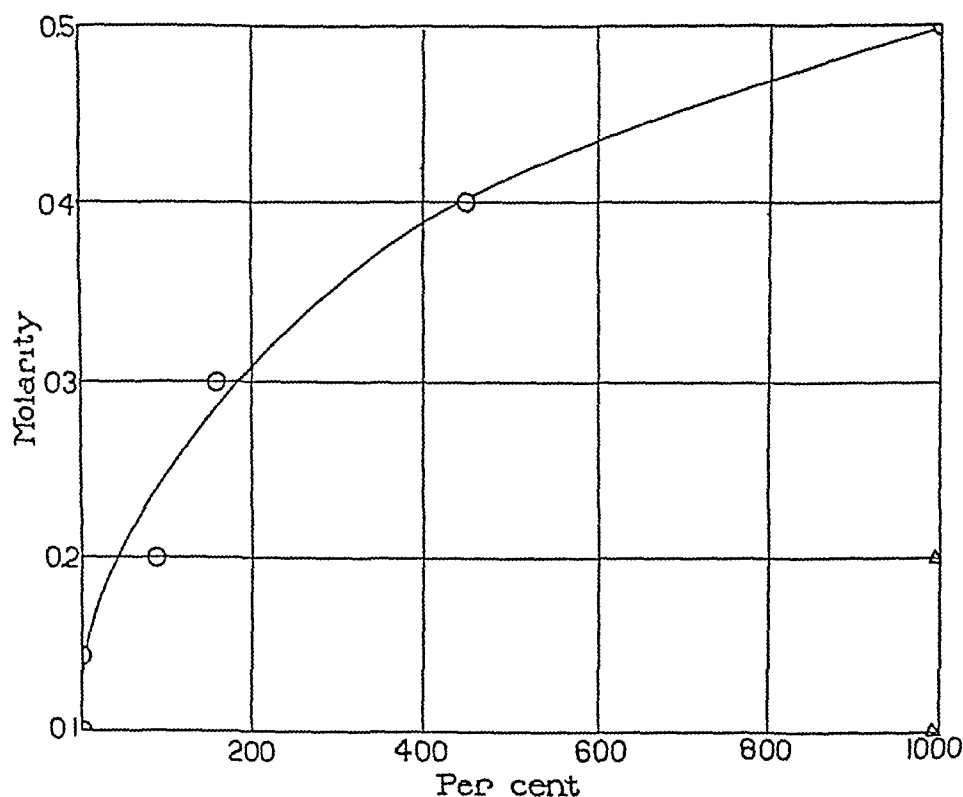


FIG 4 The effect of increasing  $[NaCl]$  on the end titre of a phage-bacteria mixture  $[P]_0 = 1 \times 10^6$  activity units/ml,  $[B]_0 = 2.5 \times 10^7$  B/ml. Mixtures maintained at  $36^\circ C$

TABLE V

*Effect of 0.25 M NaCl on Phage Distribution between Bacteria and Broth*

Mixture of resting bacteria and phage left 0.75 hr at  $0^\circ C$

$[B]_0 = 1 \times 10^8$  B/ml

$[P]_0 = 1 \times 10^9$  phage units/ml

Mixtures titrated for total phage and extracellular phage/ml

	Extracellular phage, per cent	
	Control	0.25 M salt
1	5.6	7.9
2	7.9	17.0
3	4.0	8.0
4	6.0	14.0
5	13.0	16.0
6	4.0	4.0

sponding delay in lysis and an increase in the titre of the lysate. However, neither of these effects is strictly proportional to salt concentration. Corresponding increases in  $\text{Na}_2\text{SO}_4$  produce greater delays in time of lysis (Figs 3 and 4).

7 The effect of  $\text{NaCl}$  on phage distribution between resting cells and the medium was tested by keeping a mixture containing  $1 \times 10^9$  phage units per ml and  $1 \times 10^8$  bacteria per ml for 0.75 hour at  $0^\circ\text{C}$ . The suspension was then centrifuged and determinations were made.

TABLE VI

*Effect of Adding Salt to a Phage Bacterial Mixture Just Prior to Lysis*

[B] =  $2.5 \times 10^7$ /ml

[P] =  $1 \times 10^8$  units/ml

1.5 ml. 1 M  $\text{NaCl}$  added to test series at varying times with reference to the control end point and replaced at  $37^\circ$  to lyse. 1.5 ml. normal saline added to control mixtures instead of 1 M  $\text{NaCl}$ .

Controls

1 Salt, 0.25 M  $\text{NaCl}$  present initially

2 No salt present initially

Time before control end point at which salt solution was added	Titres (hrs)		Increase in titre over control lysate
No salt added	Control	2.2	
0.25 M $\text{NaCl}$ present initially	Salt	2.7	5x
[0.9	Control	2.23	
	Salt	2.68	3.2x
0.6	Control	2.23	
	Salt	2.53	3.2x
0.3	Control	2.23	
	Salt	2.38	1.6x

of total phage and extracellular phage per ml. As shown in Table V and Fig. 1 no change in phage distribution was detected.

8 When 5 ml. portions of phage bacterial mixtures initially containing  $2.5 \times 10^7$  B/ml and  $1 \times 10^8$  phage units/ml are allowed to grow in the water bath shaker and 1.5 ml. of 1 M  $\text{NaCl}$  are added at varying times before the end point of lysis of a control mixture, lysis is delayed and the titre of the lysate is increased. The earlier the salt is added the greater is the effect (Table VI).

9 Attempts were made to extract from bacteria a catalytic agent



hypothetically responsible for the observed increase in phage titre. No such substance could be extracted by the procedures outlined below.

*a*  $2.5 \times 10^7$  *B*/ml are grown for 2.6 hours in the presence of 0.25 M NaCl and without increased salt concentration. Both bacterial suspensions are then added to 5 ml phage, iced to prevent growth of

TABLE VII

*Test for a Hypothetical Activator of Phage Extracted from Bacteria by NaCl*

5 ml of  $2.5 \times 10^7$  *B*/ml grown 2.6 hrs in presence of 0.25 M NaCl and without NaCl, iced 1 hr, added to 5 ml of phage,  $1 \times 10^{10}$  phage units per ml. Iced 1 hr. Centrifuged. Titrated for total and extracellular phage.

	Total phage log [P]	Extracellular phage log [P]	Extracellular phage
			<i>per cent</i>
Control	9.7	9.1	26
0.25 M NaCl	9.7	9.1	26
Control	9.6	8.6	10
0.25 M NaCl	9.6	8.6	10

TABLE VIII

*Test for a Hypothetical Activator of Phage Extracted from Bacteria by NaCl*

Two 5 ml aliquots of  $5 \times 10^9$  *B*/ml each left 2 hrs at 0°C in 0.25 M NaCl and in physiological saline. Added to 5 ml phage. Iced 1 hr. Titrated for total and extracellular phage.

	Total phage log [P]	Extracellular phage log [P]	Extracellular phage
			<i>per cent</i>
Control	9.7	8.7	10
0.25 M NaCl	9.7	8.7	10
Control	9.4	8.6	16
0.25 M NaCl	9.5	8.6	11

organisms, and after 1 hour of contact the mixtures are titrated for total phage/ml and extracellular phage/ml (Table VII).

*b*  $5 \times 10^7$  *B*/ml are left in 0.25 M NaCl without growth occurring and are then treated as in *a* (Table VIII).

*c*  $1 \times 10^8$  *B*/ml are left 24 hours in salt and in salt and glycerine mixtures, centrifuged, and the supernatant added to an equal volume of phage and titrated (Table IX).

10 In order to determine whether the effect at the lytic threshold was primarily on the phage or on the organisms, phage bacterial mixtures were grown with and without salt, centrifuged, the supernatants interchanged 0.45 and 0.9 hour before lysis in the control mixture, and they were then replaced to lyse at 37°C. No consistent results were obtained. Since the mixtures were either at the lytic

TABLE IX

*Test for a Hypothetical Activator of Phage Extracted from Bacteria by NaCl*

5 ml of  $1 \times 10^9$  B/ml left at 0°C 24 hrs in the following mixtures

A 0.8 M NaCl

B 0.6 M NaCl, 50 per cent glycerine

Centrifuged 1/2 hr 1 ml of the supernatant added to 1 ml of phage  $1 \times 10^{10}$  units/ml Left 1 hr at 0°C Titrated for total phage

	log [P]	
	Exp. 1	Exp. 2
0.8 M NaCl	9.5	9.6
A. Control (normal saline)	9.6	9.7
0.6 M NaCl	9.9	9.7
B. Control (normal saline)	9.9	9.7

TABLE X

*Effect of Low Temperature on Time of Lysis and End Titre of Lysate*

	37 C.	0 C.
Time of lysis		
0.25 M NaCl	2.6	4.1
Control	2.05	2.6
Titre of lysate log [P]		
0.25 M NaCl	10.75	8.5
Control	10.15	9.6

threshold or just starting to lyse, it was difficult to obtain samples in exactly the same condition each time. However, there were some indications that for the maximum effect the salt must act on phage and bacteria together at the threshold. Organisms grown in salt and added to control supernatants, for the most part did not show any changes. If the interchange was made nearer the end point of lysis

of the control, the results were very erratic, sometimes showing a slight increase in titre of the lysate and other times none at all

11 Gram stains of organisms during the period of lysis in phage-salt mixtures show no deviations from similar preparations containing no salt

12 When mixtures of phage and bacteria made with 0.25 M NaCl and without salt are grown at 36°C to within 0.4 hour of lysis and are

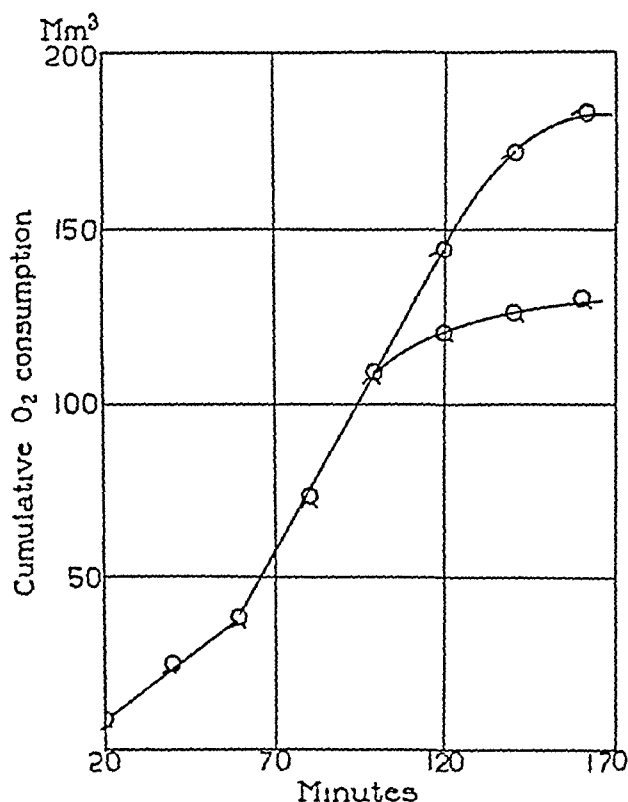


FIG. 5. Cumulative oxygen consumption of organisms grown in the presence of phage with and without salt.  $[B]_0 = 1 \times 10^8$  B/ml,  $[P]_0 = 5 \times 10^6$  activity units/ml. Temperature—36°C. Q = mixture without salt, O = mixture with 0.25 M NaCl.

then removed to a water bath at 4°C, lysis occurs in the salt mixture as much as 5 hours later than in the control. Both mixtures exhibit a tenfold diminution in end titre (Table X).

13 Organisms grown in the presence of  $5 \times 10^6$  phage units per ml showed the same rate of oxygen consumption as the controls grown without phage. The rates differ only during the period of lysis as would be anticipated. The rate of oxygen consumption during

growth in the presence of 0.25 M NaCl does not differ from the normal rate. The same holds true of organisms exposed to both 0.25 M NaCl and  $5 \times 10^4$  phage units per ml during growth.

#### DISCUSSION

The foregoing analysis of the influence of 0.25 M NaCl on the kinetics of the phage bacterium reaction leads to the conclusion that the lytic threshold is increased five to tenfold during a period just preceding the end point of lysis in the salt mixture and that the titre of the resulting lysate is five to tenfold greater than that of the control.

Lysis occurs about 0.7 hour later than in the mixture containing no salt and it is during the prolonged maximal growth stationary phase preceding lysis that the increase in the total phage concentration develops. The extent of delay in lysis caused by salt and the increase in the titre of the lysate rise with increasing salt concentration over a small range and then attain a maximum. Turbidity measurements and the rates of oxygen consumption of the bacteria during growth and phage production in the presence of 0.25 M NaCl indicate that there is no growth of organisms taking place during the period of phage production at the lytic threshold. This finding is of considerable significance since previous work has emphasized bacterial growth as an essential conditioning factor for phage production (20-24). If bacterial growth is really requisite for phage production, the only way in which phage could be produced while the bacterial growth curve remains flat would necessitate growth of young cells and an exactly equivalent lysis of older cells. The data on oxygen consumption would rule out this possibility so apparently there exist conditions under which phage production can occur in the absence of bacterial growth.

We have attempted to use other compounds in investigating specific ion and osmotic effects on bacterial growth and phage production. Unfortunately there are inherent limitations in this approach, for example the salts of trivalent anions and cations and other salts of univalent and bivalent ions either inhibit growth of the staphylococcus or form insoluble compounds with the constituents of the medium at the pH necessary for growth. Hexoses which are sufficiently soluble are fermented by the staphylococcus and other carbohydrates which are not fermented are not sufficiently soluble.

## CONCLUSIONS

1 The presence of 0.25 M NaCl during the reaction between a staphylococcus phage and susceptible organisms results in a five to tenfold increase in the amount of phage produced

2 Analysis of the reaction indicates that normal kinetic relationships exist until just before lysis occurs. At this time the organisms enter the stationary phase, lysis is delayed approximately 0.7 hour as compared with control mixtures and phage continues to be produced at the usual rapid rate

3 Apparently there are conditions under which phage can be produced in the absence of bacterial growth although previous work has uniformly emphasized growth of the bacterial substrate as the prime conditioning factor for formation of phage

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# CRITICAL ILLUMINATION AND FLICKER FREQUENCY IN RELATED FISHES

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## I

The relationships between flicker frequency and mean critical illumination for response to a moved stripe pattern have been determined for the sunfish *Leiostichus* (Wolf and Zerrahn Wolf, 1935-36 *b*, Crozier, 1935-36, Crozier, Wolf, and Zerrahn Wolf, 1935-36 *b*, *d*). When  $\log I_m$  is expressed as a function of  $F$  the curve is a double S-shaped affair, each part is describable with excellent fidelity by a logistic (or by a probability integral). The curve is not describable by the equation which has been employed to graduate the data of human flicker fusion (Hecht and Verrijs, 1933-34, Hecht, Schlaer, and Smith, 1935), the behavior of the curve as a function of temperature is also inconsistent with the stationary state conception of the basis for the shape of the graph (Crozier, Wolf, and Zerrahn Wolf, 1935-36 *c*, *d*). By means of the logistic equation for independent rod and cone contributions, the two parts of the composite flicker recognition curve have been separated (Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *d*). To test the propriety of this procedure, and also to examine further the nature of the variability of the critical illumination, it was planned to obtain the  $F-I_m$  curve for several other kinds of fresh water teleosts. Suitably chosen hybrids of these forms might then be tested. The expectation was that the flicker recognition curves would not be the same in position or in shape in the different fishes. It could then be ascertained whether the same method of dissecting the curve into rod and cone contributions gives a rational result of the same sort in the several cases. The possibility obtains that a genetic basis could be indicated for the differences to be discovered. Similar considerations apply to the data of variation of performance in the response to flicker.

Three additional forms have been carefully examined by the procedure described in our previous papers, these and the *Enneacanthus* (*Lepomis*) earlier studied provide four quite different curves in which  $\log I_m$  is given as a function of  $F$ . The analysis of these data, given in the present paper, appears to provide a very precise kind of confirmation of the correctness of the proposals we have already made in our treatment of the data on *Enneacanthus*.

The flicker response property of a given animal can be measured or expressed only by defining this property in terms of a system of coordinates—it is the shape and position of the whole curve which is significant. Properties of the curve as a whole alone will permit comparisons of flicker sensitivity among different forms. The curve is reproducible among individuals of the same type, and differs in different types (species), it is therefore a constitutional property. The adequacy of a formulation of the shape of the curve, or of a method of interpreting its shape, can therefore be tested by means of experiments designed to reveal whether elements essential to the proposed interpretation behave in inheritance as other constitutional properties are known to do. The general situation has already been developed in the similar analysis of the geotropic performance of rats (Crozier, 1929, 1935, Crozier and Pincus, 1929–30 *a, b*, 1931–32 *a, b*, 1935–36). This may very well involve, of course, something more than simple matters of dominance and single factor differences.

The fishes involved<sup>1</sup> were (a) a clonal strain of the swordtail ("Green Helleri") *Xiphophorus helleri*, (b) a clonal strain of the Black Platy ("Golden Helmet"), *Platypoecilus maculatus*, and (c) an inbred strain ("Black Helleri") derived from  $F_1(a) \text{ } \varnothing \times (b) \text{ } \sigma^7$  by backcrossing fertile  $F_1$  females to (a)  $\sigma^7$ . The Black Helleri stock is uniform and stable. (d) In addition, a clonal strain of Red Platy (*P. maculatus rubra*) has been available for comparison, as well as (e) a stock of *P. variatus* and (f) the  $F_1$  generation hybrids between (a) and (c). We are under obligation to Dr. C. P. Haskins for assistance in obtaining these stocks. We have also tested (g) another species of sunfish, *Lepomis gibbosus*, for comparison with our *Enneacanthus*. The possibility exists that different species and lines of *Platypoecilus* and

<sup>1</sup> For data of the status and interrelations of these types cf. Bellamy (1924), G. C. C. (1927, 1931), and Fraser and Gordon (1929).

of sunfish, as well as others, may exhibit essentially similar flicker response curves, with or without significant differences in the variability of performance. Changes encountered in the progeny of species crosses would then be of added significance.

Our main purpose has been to discover whether (1) different flicker recognition curves would be encountered in the several species, and if so whether (2) the forms of the specific curves could be modified as result of crossing. We are not in position to discuss the mode of inheritance of the changes actually found, with slightly different material which it is proposed to utilize subsequently, it may be possible to do this with some detail and precision. Without being required, however, to provide a scheme of a mechanism of factorial inheritance in this case, we do obtain evidence which definitely points to the hereditary determination of properties of the curve of critical illumination as a function of flicker frequency. There is also secured a rather neat justification of our procedure for the separation of rod and cone components of the curve.

## II

### PROCEDURE

The observational procedure was that previously described in detail (Wolf and Zerrahn Wolf, 1935-36*b*; Crozier Wolf, and Zerrahn Wolf, 1935-36*a, b, c, d*). Determinations of critical illumination were made at fixed flicker frequencies  $F$  at temperature 21.5°C. For each type of fish the same 10 numbered individuals were used throughout the tests. At each  $F$  3 readings were taken on each individual, previously dark adapted. The average of these was taken as  $I_1$ , the mean of the 10  $I$ 's =  $I_m$ , from which  $P E_{I_1}$  was computed.

The basis of the measurements resides in the behavior of the fish. With any one form the variation in  $I_1$  among individuals is much greater than among the three measurements on each fish (except at  $I_m$ ). The values of  $P E_{I_1}$  therefore enable one to study the variation of performance as a function of intensity,  $F$ , species and ultimately of other variables. The variation of performance is not a matter of persistent individual differences within a given stock. The relative sensitivities of the 10 individuals in one stock in any one test may be expressed by rank order numbers in the sequence of increasing intensities required to obtain response. These relative sensitivity positions show no correlation in successive tests. The mean rank order numbers are distributed quite at random (Table I). This corresponds in all details with what we have already learned in our measurements with the sunfish (Crozier Wolf, and Zerrahn Wolf, 1935-36*b, d*). The basis for this interpretation of the variation of  $I_1$ , and for the method of averaging



employed, has been tested in several ways. The presumption is, that by taking three successive readings with each individual fish (a) the chance of gross error is reduced, (b) disturbances possibly introduced by handling the fish and transferring it to the observation chamber are minimized and, (c) the average of three such readings should reduce the error of estimation of  $I_c$ , the critical illumination, be-

TABLE I

A summary of mean rank orders of sensitivity in sets of measurements with three types of fishes, showing the chance distribution of relative sensitivities in each type, as seen in successive sets of measurements

	Mean of individual average rank order Nos.	Maximum departure of an individual mean	Differences between extreme individual means
<i>Xiphophorus helleri</i> (31 sets, $n = 10$ individuals)	5.47 ± 0.490	$2.86 \times P E_1$	$2.09 \times \sigma_{d,f}$
<i>Platypoecilus maculatus nigra</i> (22 sets, $n = 10$ )	5.50 ± 0.659	$2.69 \times P E_1$	$3.0 \times \sigma_{d,f}$
Black Hellen (hybrids) (21 sets, $r = 10$ )	5.50 ± 0.917	$2.75 \times P E_1$	$3.1 \times \sigma_{d,f}$

TABLE II

Comparison of variation of  $I_1$  as obtained in parallel tests with a set of ten Little Sunfish (*Eupomotus gibbosus*) at two flicker frequencies ( $F$ ) in which (A) each  $I_1$  is the average of three successive readings with each individual and (B) the readings are taken in three successive series of one observation on each fish,  $I_m$  is not significantly different in A and B, but in the latter case  $P E_1$  is slightly but significantly lower. See text.

$F =$	6	35
	$\log I_m \quad \log P E_1$	$\log I_m \quad \log P E_1$
Method A	$\bar{5} 2378 \quad \bar{7} 7837$	$0 1335 \quad \bar{2} 3282$
Method B	$\bar{5} 2435 \quad \bar{7} 5680$	$0 1345 \quad \bar{2} 2028$

cause the fish will be in about the same intrinsic reactive state. If instead of following this procedure, three sets of single readings on each fish are taken in succession,  $P E_1$  calculated in the same way should then be less than that obtained for the same individuals under the same conditions by the first process, although larger if calculated from the mean of the 30, because the reactive condition of each fish presumably fluctuates more in the longer period over which its

readings are spread—the observations tend therefore to be less clumped in their distribution. Results from such a test are given in Table II. The fishes used were 10 Little Sunfish (*Eupomolus gibbosus*) they were examined by the routine method at four flicker frequencies (cf Table III) and then at two of these by the method of testing each fish in succession, running through the set 3 times between tests each fish was in the thermostat in the dark. Analysis by variance test of the individual records shows the same kind of variation among successive readings on one fish as among equivalent readings on different fishes, whereas by our standard procedure the variation is predominantly from fish to fish, although this variation

TABLE III

Comparisons of mean critical illuminations ( $I_m$ ) at 21.5°C for various types of fishes at several flicker frequencies ( $F$ ), showing that the various pure types of *Platyocilus* agree as regards  $\log I_m$  and  $\log P E_{I_1}$  (cf Fig 1) and that two species of sunfish agree as concerns  $I_m$ , but differ significantly as to  $P E_{I_1}$

Species	$F$				
	6	9	20	30	35
Black Platy ( $n = 10$ )	$\bar{3} 2480$ $\pm \bar{3} 8606$	$\bar{1} 3535$ $\pm \bar{3} 9244$	$0 1216$ $\pm \bar{2} 5050$	$0 4553$ $\pm \bar{1} 0077$	
Red Platy ( $n = 4$ )	$\bar{3} 2721$ $\pm \bar{3} 8392$	$1 3699$ $\pm \bar{3} 9112$	$0 1415$ $\pm \bar{2} 7622$	$0 4704$ $\pm \bar{2} 6522$	
<i>P variatus</i> ( $n = 4$ )	$\bar{3} 2725$ $\pm \bar{3} 9240$	$1 3608$ $\pm \bar{3} 7337$	$0 1248$ $\pm \bar{2} 5112$	$0 4643$ $\pm \bar{2} 5606$	
Sunfish <i>Enneacanthus</i> * ( $n = 12$ )	$\bar{5} 2385$ $\pm \bar{6} 4814$	$\bar{3} 7983$ $\pm \bar{3} 0934$	$\bar{1} 2591$ $\pm \bar{2} 2480$		$0 1418$ $\pm \bar{1} 1106$
Little Sunfish <i>Eupomolus</i> ( $n = 10$ )	$\bar{5} 2378$ $\pm \bar{7} 7837$	$\bar{3} 7835$ $\pm \bar{4} 0969$	$\bar{1} 2620$ $\pm \bar{3} 5519$		$0 1335$ $\pm \bar{2} 3282$

\* Data from Wolf and Zerrahn Wolf, 1935-36a, and Crozier 1935-36

is randomly distributed as regards the individuals when the whole lot is repeatedly tested (Crozier, Wolf, and Zerrahn Wolf, 1935-36 a c d). In the second case, as expected,  $I_m$  is identical but  $P E_{I_1}$  is definitely and significantly less than in the first, but the rectilinear relationship between  $P E_{I_1}$  and  $I_m$  is not distorted. The sunfish has for this test the convenient advantage that the latitude of scatter of  $P E_{I_1}$  (a measure of  $\sigma_{P E_{I_1}}$ ) is less (at this temperature) than with several of the other forms we have studied.

The response upon which the measurements rest is a swimming movement in which the fish follows the rotating stripes. The critical illumination,  $I$  is the threshold intensity obtained by smoothly increasing the intensity from a very

low level until the fish just begins to move with the revolving vertical stripes (Description of apparatus in Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *a, b*) Quick, abrupt increases of illumination may give a "shock reaction" which has nothing to do with the responses indicating discrimination of light and dark bars Fishes used in the experiments are kept in the laboratory for a certain length of time, under a regular regime, in aquaria each containing a single individual Not infrequently the first set of measurements is more irregular, and its mean less reproducible, than is true with subsequent sets For this reason, and to check the possibility of progressive changes due to alterations of behavior of the fishes, or to education of the observer in the operations of measurement with them, the readings are taken in a random succession of magnitudes of the fixed flicker frequencies; and it is arranged that duplicate sets of determinations are made at certain points The values of  $I_m$ , the mean critical intensities, and the associated indices of dispersion of  $I_1$ , provide a basis for evaluating the inner coherence of the data obtained with each type of animal used

The observations, and their dispersions, may be influenced by (1) properties of the fishes and (2) by properties of the observer and apparatus, the relation between (1) and (2) determines the recorded measurements Observers and apparatus being the same, the functioning of the observer may nonetheless be influenced in an important way by specific differences in the deportment of the fishes The types used in the experiments now discussed show characteristic differences in behavior, not correlated with sex These differences are important for the interpretation of the variation data (sections III and V)

*Xiphophorus* was hyperactive, "jumpy," in the first trials, and very reactive to minor vibrations in the apparatus After several days of preliminary tests they were quieter The reaction at a critical illumination is sharp and clear, the fish swims with the moving stripes, except quite rarely when there occurs a backward motion, opposite in direction Frequently responses cease after a first "jump," even if the intensity is increased beyond the threshold, the fish then stays close to the wall of the container, with a sinuous flexure of the body and rapid fin movements On the whole, the responses were "better," from the standpoint of the observer, at low flicker frequencies than at high

turning with the moving stripes and at the same speed. This behavior is like that of the sunfish.

Black Helleri—the hybrid type previously described—shows at first the jumpiness characteristic of the swordtail but the reactions at threshold illumination for response to flicker are later less sharp. The general behavior is much like that of the Platys.

The Little Sunfish (*Eupomotus gibbosus*) is really indistinguishable in its reactions from *Enneacanthus* (Wolf and Zerrahn Wolf, 1935-36b; Crozier, Wolf, and Zerrahn Wolf, 1935-36a).

These characteristic differences must be expected to play some part in the determination of the dispersions of  $I$  and of  $I_1$ , the extent of their significance can not be precisely foreseen, and remains to be examined experimentally, as we have already remarked (Crozier, Wolf, and Zerrahn Wolf, 1935-36d). The indication thus far is that differences such as those recorded are of relatively minor significance. This becomes of special interest when the dependence of  $P/E_1$  upon  $I_m$  (or the reciprocal dependence) is considered for various forms (section V).

### III

For the purposes of our analysis it was desirable to ascertain (1) if fishes of the same generic type give similar flicker response curves and (2) if fishes of types quite different structurally and ethologically give flicker responses which are quantitatively diverse. The major portion of this paper is occupied with the second point. The physiological significance and the presumptive utility of the establishment of diverse flicker response curves for genetically disparate stocks, including the weight to be placed upon the results of genetic tests, in a sense depends upon the outcome of attempts to discover if genetically similar types provide rather closely comparable flicker curves. The phrase "genetically similar types" must be understood as used in a broad, loose sense—in the sense, namely, that varieties and species of *Platyphocilius* for example are more like one another than they are like the swordtails or the sunfishes. Changes in the flicker response curve which appear, then, in progeny resulting from the cross breeding of two contrasting types cannot be regarded as induced by minor or secondary genetic differences, or as superficial accidents, but must be interpreted as due to rather deep-seated and significant processes genetically determined.

We have compared the critical illuminations for response to flicker at four spaced flicker frequencies, two in the rod portion of the curve and two in the cone portion, for three types of Platy (including two species as ordinarily recognized), and two species of sunfish. The results are given in Table III. It is apparent that there are minor and doubtfully significant differences in the values of  $I_m$  among the three Platy forms, and only small differences between the values for the two sunfishes. The variation data we will deal with in a later section. The general concordance of the measurements for the Platy forms is such as to indicate comparative uniformity in the character of the respective flicker response curves.

and the same is true of the two sunfishes. It is to be understood that more complete determinations of the forms of the curves, like those obtained for the other species we are to consider, would very probably establish minor varietal and specific differences, but in contrast to the differences between the curves for sunfishes, Platys, and swordtails, such differences are of slight consequence. The indication is that the intratype differences appearing in Table III are comparable to the changes brought about in one individual by altering the temperature (Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *c, d*). They might be obscurely correlated with ethological factors, or even determined by the sizes of the eyes. The generic differences between flicker curves with which we shall have to deal are of a different order.

#### IV

We have determined with all possible care, and over the greatest manageable range of flicker frequencies, the curve of  $I_m$  as a function of  $F$  for a group of *Xiphophorus helleri* ( $X$ ), another of *Platypoecilus maculatus nigra* ( $P$ ), and for the Black Helleri hybrids. The latter had been obtained from backcrosses of certain  $F_1$   $X \times P$  females to  $X$  males (see section II). Each group contained 10 individuals, in each case the same 10 individuals were used throughout, no individual differences appeared in a group (section II). The results are collected in Table IV. We shall discuss first the curves for *Xiphophorus* and *Platypoecilus* comparing them with our curves for the sunfish *Enneacanthus* (Wolf and Zerrahn-Wolf, 1935-36 *b*, Crozier, 1935-36, Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *a, d*), the curve for the hybrids is considered in section VI.

Fig. 1 shows certain very definite differences among these three flicker response curves. The differences are not obliterated if the flicker frequency is in each case plotted as a percentage of the respective maximum  $F$ , or if intensity is so considered. The differences involve matters of rod and cone  $F_{raz}$ , slopes of cone and rod portions, and position on the  $F$ - $I$  grid.

We have shown that at 21.5° the curve for *Enneacanthus* can be, as to its cone portion, fairly well described by the equation which has been used to describe human flicker fusion data (Hecht, Schlaer, and Smith 1935) but that the fit is not really adequate and in particular systematically fails at other temperatures (Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *d*). This equation (Fig. 2) also fits fairly well

TABLE IV

Mean critical illuminations,  $I_m$  (millilamberts) for response to flicker, as a function of flicker frequency ( $f$ ), in homogeneous groups of *Asphosphorus*, *Platypocilius* and extracted backcross hybrids of these (Black Hellen, *H*), with the probable errors (P E  $I_1$ ) of the dispersions

$f$ per sec	<i>Asphosphorus helleri</i>		<i>Platypocilius maculatus nigra</i>		Hybrids	
	$\log I_m$	$\log P E I_1$	$\log I_m$	$\log P E I_1$	$\log I_m$	$\log P E I_1$
1			$\bar{6}$ 2911	$\bar{8}$ 8502		
2	$\bar{7}$ 9379	$\bar{8}$ 5393	$\bar{6}$ 8445	$\bar{7}$ 2472	$\bar{6}$ 4690	$\bar{7}$ 0185
3	$\bar{6}$ 3420	$\bar{7}$ 0969	$\bar{6}$ 3579	$\bar{7}$ 9183	$\bar{6}$ 9547	$\bar{7}$ 5827
4	$\bar{6}$ 6052	$\bar{7}$ 2299	$\bar{6}$ 9042	$\bar{6}$ 1386	$\bar{6}$ 3906	$\bar{7}$ 9760
5	$\bar{6}$ 8193	$\bar{7}$ 4324	$\bar{4}$ 6977	$\bar{5}$ 2485	$\bar{4}$ 2669	$\bar{5}$ 0784
	$\bar{6}$ 8172	$\bar{7}$ 2250	$\bar{4}$ 8911	$\bar{5}$ 2558		
6			$\bar{3}$ 2470	$\bar{5}$ 7999	$\bar{4}$ 9440	$\bar{5}$ 5780
	$\bar{6}$ 3721	$\bar{7}$ 9653	$\bar{3}$ 2489	$\bar{5}$ 9212		
	$\bar{6}$ 2826	$\bar{7}$ 7148				
	$\bar{6}$ 3539	$\bar{7}$ 8341				
7	$\bar{6}$ 8399	$\bar{6}$ 2390	$\bar{2}$ 1367	$\bar{4}$ 3115	$\bar{3}$ 4603	$\bar{4}$ 2074
	$\bar{6}$ 6078	$\bar{7}$ 9353				
	$\bar{6}$ 6964	$\bar{7}$ 9857				
8			$\bar{2}$ 8251	$\bar{3}$ 3579	$\bar{2}$ 0546	$\bar{4}$ 5320
	$\bar{4}$ 3191	$\bar{6}$ 9312				
	$\bar{4}$ 3266	$\bar{5}$ 0042				
9	$\bar{3}$ 2469	$\bar{5}$ 8295	$\bar{1}$ 3535	$\bar{3}$ 9244	$\bar{2}$ 6316	$\bar{3}$ 1471
	$\bar{3}$ 2378	$\bar{5}$ 8374				
10	$\bar{2}$ 3164	$\bar{4}$ 8594	$\bar{1}$ 5967	$\bar{2}$ 2185	$\bar{2}$ 8913	$\bar{3}$ 4250
12	$\bar{2}$ 6651	$\bar{3}$ 0909			$\bar{1}$ 3296	$\bar{2}$ 0065
15	$\bar{2}$ 8918	$\bar{3}$ 2650	$\bar{1}$ 9056	$\bar{2}$ 2253		
16					$\bar{1}$ 6567	$\bar{2}$ 1072
20	$\bar{1}$ 1900	$\bar{2}$ 1055	$\bar{0}$ 1216	$\bar{2}$ 5050	$\bar{1}$ 9376	$\bar{2}$ 5064
	$\bar{1}$ 1772	$\bar{3}$ 3939	$\bar{0}$ 1106	$\bar{2}$ 5552	$\bar{1}$ 9330	$\bar{2}$ 2862
25	$\bar{1}$ 4708	$\bar{3}$ 9719	$\bar{0}$ 2911	$\bar{2}$ 8185	$\bar{0}$ 1741	$\bar{2}$ 8874
	$\bar{1}$ 4598	$\bar{3}$ 9677				
30	$\bar{1}$ 7571	$\bar{2}$ 1169	$\left\{ \begin{array}{l} \bar{0} \text{ 3838} \\ \bar{0} \text{ 4653} \end{array} \right\}$	$\left\{ \begin{array}{l} \bar{2} \text{ 9631} \\ \bar{1} \text{ 0077} \end{array} \right\}$	$\bar{0}$ 4502	$\bar{2}$ 8120
35	$\bar{0}$ 1520	$\bar{2}$ 9002	$\bar{0}$ 6676	$\bar{2}$ 9849	$\left\{ \begin{array}{l} \bar{0} \text{ 8445} \\ \bar{0} \text{ 8354} \end{array} \right\}$	$\left\{ \begin{array}{l} \bar{1} \text{ 8874} \\ \bar{1} \text{ 0849} \end{array} \right\}$
38	$\bar{0}$ 4488	$\bar{2}$ 9271				
40	$\bar{0}$ 7213	$\bar{1}$ 2224	$\bar{1}$ 0390	$\bar{1}$ 0637	$\bar{1}$ 4065	$\bar{0}$ 0916
42	$\bar{1}$ 3057	$\bar{1}$ 5398			$\bar{2}$ 1691	$\bar{0}$ 3228
	$\bar{1}$ 2785	$\bar{1}$ 5864				
42.5					$\bar{2}$ 2838	$\bar{0}$ 3581
43	$\bar{2}$ 2174	$\bar{0}$ 2309	$\bar{1}$ 3290	$\bar{1}$ 6012		
	$\bar{2}$ 2177	$\bar{0}$ 3955				
46			$\bar{2}$ 2953	$\bar{0}$ 1021		

## CRITICAL FLICKER FREQUENCY IN FISHES

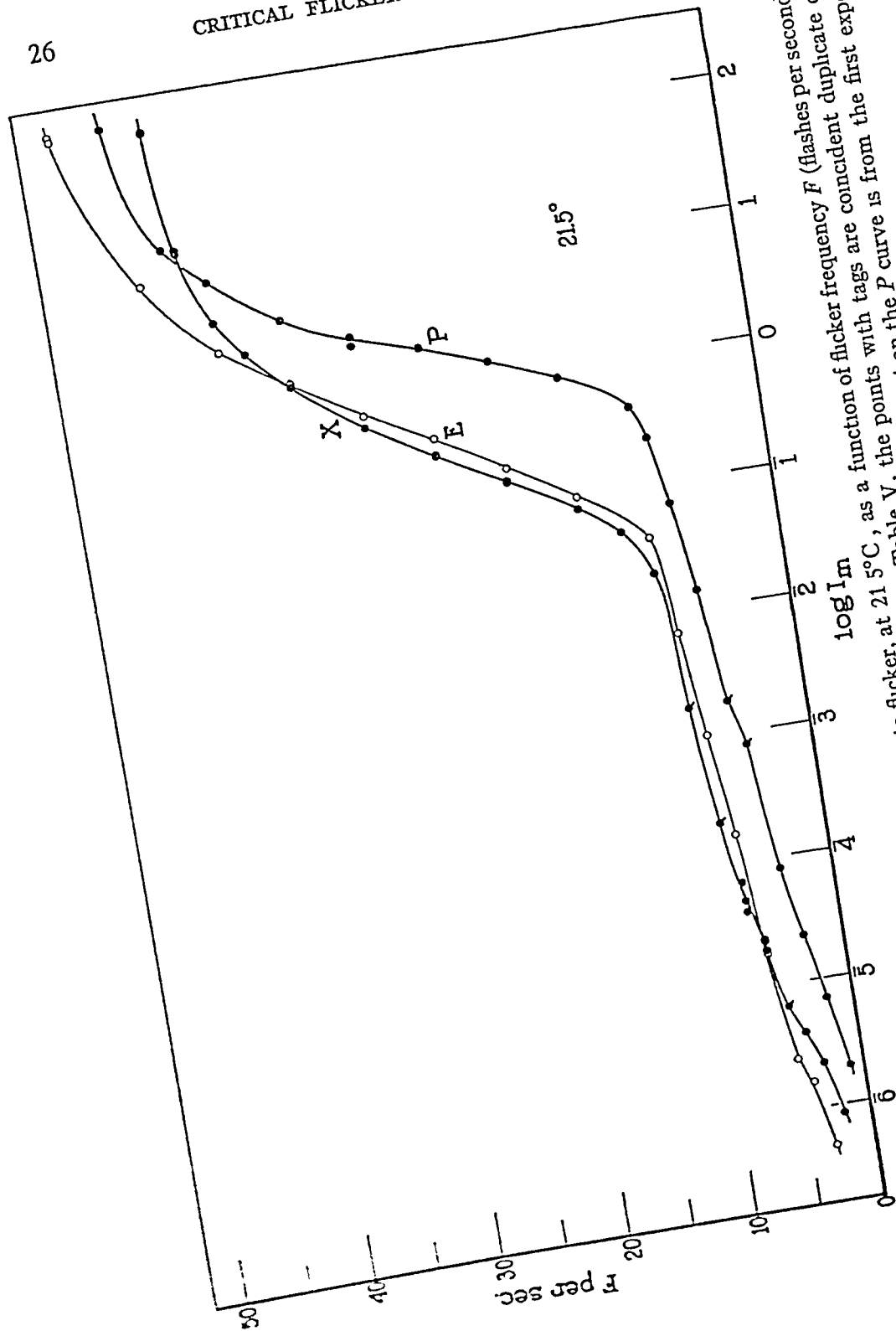


FIG. 1 Mean critical illumination  $I_m$  for reaction to flicker, at  $21.5^\circ\text{C}$ , as a function of flicker frequency  $F$  (flashes per second), for *Xiphophorus* (X), *Platybocellus* (P), and *Enneacanthus* (E). Data in Table V, the points with tags are coincident duplicate determinations of  $I_m$ , data for *Enneacanthus* from previous papers (The one aberrant point on the P curve is from the first experiment (see Table V) with these fishes).

the data on *Lipophorus*, but cannot be used for the *Platyphacellus* data without the impossible assumption that the photochemical process is of about the 17<sup>th</sup> order. There would also be involved a

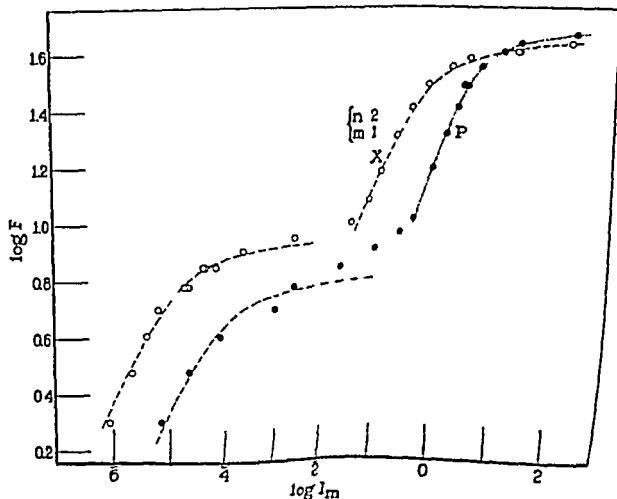


FIG. 2. Critical illumination,  $\log F$ , as a function of  $\log I_m$ , to illustrate results of fitting by the stationary rate equation (Hecht, Shlaer, and Smith 1937)  $R/F = F/(F_m - F)^n$  with  $n = 2$ ,  $m = 1$ . For *Lipophorus* (X) the fit is poor (as for *Enneacanthus* at this temperature) both with rods and cones; for *Platyphacellus* (P) the fit for the rod portion is not sufficient, while the cone part ( $\log I_m > 0$ ) would require fractional exponents. See text.



with different values of  $F_{max}$ ,  $p$ , and  $I$ , for the two parts and with a particular sort of addition of critical  $F$ 's in the region of overlap. The value of  $p$  is independent of temperature.

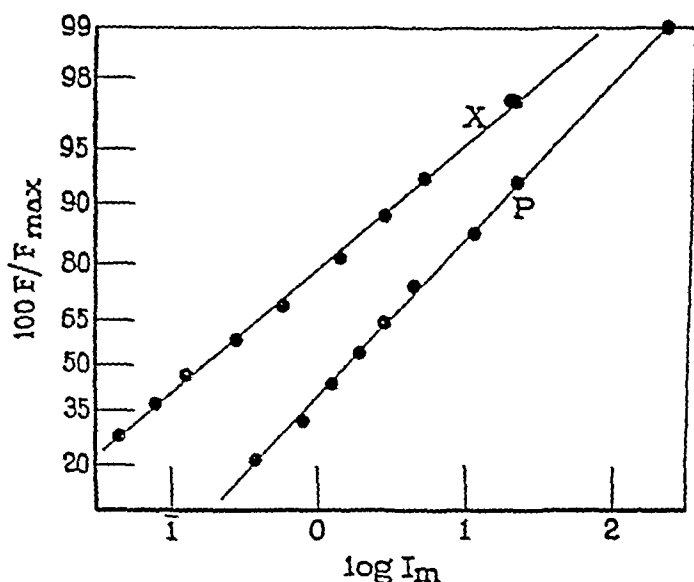
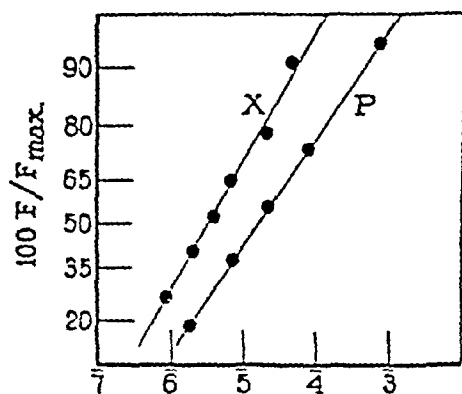


FIG 3 The upper (cone) segments of the  $F-I_m$  curves for *Xiphophorus* (X) and *Platypoecilus* (P) on a log logistic grid, these segments are presumed not to be complicated by the addition effects due to rods



hybrid group (*H*), discussed subsequently. For convenient comparison the descriptive constants for the several forms have been collected in Table V. It is apparent that there is no necessary association between values of  $F_{max}$ ,  $p$ , and  $I_t$ .

The logistic has been used for these curves partly as a matter of convenience, because it efficiently describes the data in diverse instances, because of the nature of the dispersions of  $I_1$ , because it permits testable extrapolations, because it gives a transformation in which the  $F I_m$  and  $F_m I$  curves become identical (Crozier, Wolf, and Zerrahn Wolf, 1935-36 *d*), and because it is susceptible to suggestive interpretation. In the cases to which it does not completely apply (bee, and *Anax*; Crozier, Wolf, and Zerrahn Wolf, 1935-36 *b, c*) there

TABLE V

Constants for the description of the flicker response curves of four types of fishes. The equation used is that of the logistic

$$F = F_{max} / (1 + e^{-p \log I/I_t})$$

	Rods			Cones		
	$F_m$	$p$	$I_t$	$F_m$	$p$	$I_t$
1 <i>Enneacanthus</i>	8.0	1.83	0.000 003	50.5	1.57	0.372
2 <i>Asphorhynchus</i>	7.7	1.95	0.000 003	43.1	1.90	0.177
3 <i>Platypharodon</i>	5.4	1.38	0.000 016	46.5	2.29	1.62
4 Black Heterichthys hybrid sex (2) and (3)	5.4	2.00	0.000 006	43.1	1.90	0.832

appear to be structural factors which interfere with its application at low flicker frequencies. It is to be noted, however, that a probability integral of course fits the data about as well—perhaps even better, and might ultimately prove theoretically useful.

The curves for sunfish, swordtail, and Platy (Fig. 1) differ in form, proportions, and position, although their morphology is similar. The two branches of each curve must therefore be assumed due to homologous features of the several fishes. The connection between the rod and cone parts of each curve shows distinct differences in form. This gives opportunity to test the efficacy of the method we have used to separate the rod contribution from that due to the cones (Crozier, Wolf, and Zerrahn Wolf, 1935-36 *a, d*). We have given reasons for assuming that the complete rod flicker curve, if obtainable separately,

should rise to a flat maximum and then decline to zero, the cone curve, starting at zero, is in terms of  $F$  added to that for the rods. The obvious "bump" on the flat portion of the composite curve (Fig 1) is due to the entrance of the cone contribution. If this idea be correct, the logistic extrapolation must give a consistent result with flicker curves of very different shapes. The extrapolated curves are plotted in Fig 5. They clearly do approach zero at the proper place in each case. This is also true with the hybrid curve discussed in section VI.

The decline of the rod contribution to the recognition of flicker which is contemplated in this treatment, and required by the theory

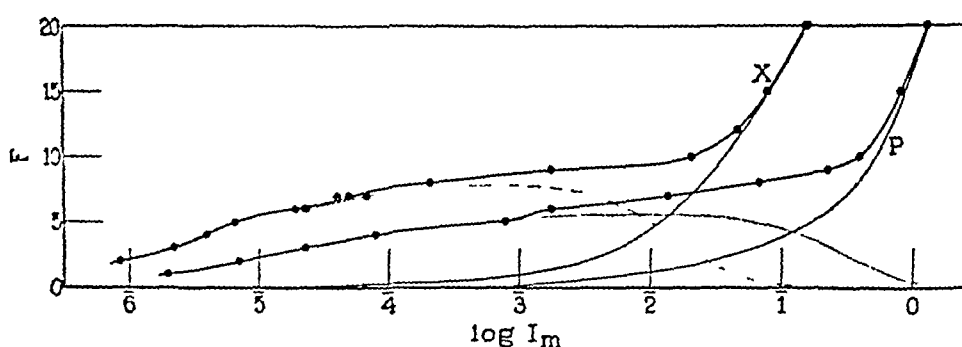


FIG. 5. The lower portions of the  $F$ - $\log I_m$  curves for swordtail (X) and Platy (P), showing the extrapolation of the logistics fitted to the cone parts (Fig 3). The difference curves are shown dotted. See text.

tested. The reality of this fluctuation has been checked by means of paired sets of observations at two flicker frequencies, one of these being in the region of the cone curve's start, the other at a nearby level. Very precise concordance was always obtained, except at  $F = 6, 7$ , and  $8$ , at  $F = 5$  or  $9$ , new determinations of  $I_m$  showed the sort of agreement which experience has demonstrated to be characteristic at other parts of the *λiphophorus* flicker curve, in that for *Platy poecilus*, in the hybrids, and in the four curves determined for *Ennea*

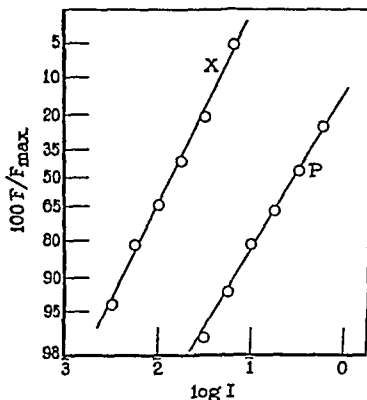


FIG 6 The difference curves computed (graphically) to depict the decline of rod contribution to the composite flicker curve (Fig 5) are described by a logistic the exponent is not the same as for the rising branch of the rod curve—for *λiphophorus* (X),  $p = 2.10$  for *Platy poecilus* (P),  $p = 3.44$

*canthus*. The differences at  $F = 6, 7, 8$  (greatest at  $F = 6$ ) are statistically significant, the scatter of the individual sets of measurements is not affected, as the plot of  $P E_{I_1}$  vs  $I_m$  shows no disturbances at this level (cf Fig 7). The fluctuation therefore signifies a real fluctuation in the threshold intensity for cone function in flicker recognition. This may be compared with day to day fluctuations in the mode of junction of rod and cone dark adaptation curves for a human eye, the intensity for threshold discrimination which supplies the measure of

directly proportional to  $I_m$ , but to  $I_m + \text{a constant}$  (or, to pass through the origin, a constant must be subtracted from the observed values of  $P E_{I_1}$ ). The intensities at which the breaks occur can be stated only approximately, in view of the breadth of the band which includes the observations, but for the three fishes these were at about

$$\begin{aligned} I_{mb} &= 0.10 \text{ millilambert for } \textit{Enneacanthus}, \\ &= 3.16 \text{ millilamberts for } \textit{Xiphophorus}, \\ &= 2.00 \text{ millilamberts for } \textit{Platypoecilus} \end{aligned}$$

The break is less clear in the *Platy* graph (in the hybrids subsequently discussed it is scarcely apparent at all), so that the magnitude of the corrective constant can be given only in a very approximate way, above the break intensities  $I_{mb}$  the values of  $P E_{I_1}$  must be diminished by

$$\begin{aligned} 0.10 &\text{ for } \textit{Enneacanthus}, \\ 0.080 &\text{ for } \textit{Xiphophorus}, \\ 0.054 &\text{ for } \textit{Platypoecilus} \end{aligned}$$

The question naturally arises as to whether the proportionality factors  $k$ , the antilog intercept constants in Fig. 7, differ significantly. We have assumed that the variability of critical illumination measures essentially a property of the reacting mechanism of the animal, and we have discussed its relation to temperature upon this basis. It may be important for such a view that at any given intensity the values of  $P E_{I_1}$  are rather similar in the various forms which have been tested. Considerations directly arising from the quantitative properties of  $P E_{I_1}$  as obtained in the measurement of diverse visual functions with the same organisms (Crozier, 1935-36, 1936), by means of tests fundamentally involving intensity discrimination in all cases, can be understood only on the assumption that the dispersions of the measurements of critical intensities, and the dependence of these dispersions upon  $I_m$ , are due to a fluctuating property of the reacting organism and not to experimental error in the ordinary sense, they are not identical at given intensity. We have noted previously (Crozier, Wolf, and Zerkow-Wolf, 1935-36 d) that in the sunfish uncontrolled conditions may introduce consistent temporary changes in  $P E_{I_1}$  without altering  $I_{mb}$  or critical frequencies, these are correlated with obvious departures from the customary mode of behavior of the fishes. At the

same time, alteration of temperature produces diverse effects upon  $PE_I$ , and upon  $I_m$ , of identical sort in two quite different organisms (Crozier, Wolf, and Zerrahn Wolf, 1935-36 *c, d*) and not correlated with obvious changes in the character of the motor response. These findings indicate that a probably interesting outcome would result from systematic attempts to modify  $PE_I$ , experimentally, such as we have pointed to in our preceding paper. In the meanwhile, however, it is desirable to review and codify the findings in our routine determinations of the variation of critical illumination for response to flicker. It is not to be lost sight of that naive applications of probability theory to such dispersions may be quite inappropriate. In an ordinary physical measurement, as of the precise length of a steel bar by micrometer setting, under continuous constant conditions, the mean of the measurements is determined by the actual length and the individual settings are made under the "restoring agency" of the observer's appreciation of an incorrectness of setting. In measurements of critical illumination or of critical flicker frequency the observer is called upon to make a setting with respect to the index response, not to make a photometric match of intensities, three such readings on an individual are averaged to give  $I_1$ , and ten  $I_1$ 's to give a mean. The mean  $I_c$  from a series of such readings has no meaning with respect to an individual determination, and  $\sigma_1$  is merely an index of scatter. A clear discussion of these matters which is unusually helpful is given by Whitehead (1934). The contributions respectively made to the total scatter by (a) the observer and the process of observation, and (b) the intrinsic variability of the reacting organism, must be disentangled by suitable experimental tests. The behavior of the readings when subjected to a variance analysis test (*cf.* section II) only partially resolves the problem. In crudest form the alternatives are (1) that the properties of  $PE_I$ , as measured are a valid reflection of properties of the fluctuation of the reacting mechanism of the reacting organism, and (2) that they depend upon properties of observer *plus* procedure. It is to be inquired if the latter notion gives an adequate interpretation of the facts.

It could be expected on the first view (although not as a *necessary* consequence) that for quite different organisms the values of  $PE_I$ , might differ markedly at a given  $I_m$ , in measurements of the same type

of visual response—particularly if the respective curves of critical illumination as a function of flicker frequency should be strikingly different. Fig. 6 might be thought to indicate that such expectation is not really substantiated. The conclusion might then be, either that  $PE_{I_1}$  reflects merely the error of observation, predominantly due to circumstances outside the reacting animal, or that, though proper to the organism,  $PE_{I_1}$  is too nearly alike in different animals to be an analytical key to differences in excitability. (It is to be remembered, however, that regardless of the mechanism of determination of  $PE_{I_1}$ , it describes an attribute of the data which governs in an important way the uses which can be made of them when comparisons are made with the requirements of an interpretive theory of the response to flicker.)

The differences visible in the variability functions for *Enneacanthus*, *Xiphophorus*, and *Platypoecilus* are real enough, in the sense that their consistency makes them quantitatively significant. But it might be that they could arise through differences in the training of the observer, so that his appreciation of the reaction signal has improved, or they might be due to small, constant, characteristic, differences in the relation of the diverse reacting animals to the observer. The first contention is scarcely reasonable, however, because in the course of a month or more of intensive work with a single reacting species there is quantitatively no change in the magnitudes of  $PE_{I_1}$  at given flicker frequencies, this has been repeatedly determined by means of reduplications of tests at the beginning and at the end of an experimental run, consequently there is no evidence that training of the observer has influenced  $PE_{I_1}$ . Moreover, this would quite fail to account for the self-consistent, characteristic properties of the spread coefficient  $k$  which measures the proportionate scatter of  $PE_{I_1}$ .

A distinction must certainly be made between (1) *variation of per-*

modify both performance and variation of performance as a function of constant external conditions. We can get a clue to the operation of the distinction between (1) and (2) in the case of our flicker experiments by considering the difference between (a) the variation of critical flicker frequency as a function of fixed intensities and (b) the variation of critical intensity as a function of fixed flicker frequencies. It has been shown that the two kinds of variation are mutually interdependent (Crozier, 1935, 1935-36, 1936, Crozier, Wolf, and Zerrahn Wolf, 1935-36 a, b). The former, (a), corresponds to fluctuations in *performance*, it has been interpreted as due directly to organic fluctuations in the mechanism basic to discrimination (by response) between the effects of flashes and the effects expressed as their after images (Crozier, Wolf, and Zerrahn Wolf, 1935-36 c). The latter, (b), corresponds to fluctuations in the frequency of a heart beat, for example, as a function of the temperature, for such fluctuations reflect variations in the potential (*intensity*) of the chemical process the velocity of which determines the interval between exhibitions of a fixed level of performance, namely the discharge of the pacemaker relaxation oscillator governing the frequency of beats (*cf* Loeb, 1900, Hoagland, 1935, Crozier, 1929).

We may consider the general properties of these two classes of variations or fluctuations, as empirically obtained in diverse instances, in order to demonstrate ( $\alpha$ ) that the relation of  $P E_{I_1}$  to  $I_m$  may be very similar, quantitatively, for diverse organisms in which the respective relations of  $I_m$  to a common independent variable may be quite unlike, and ( $\beta$ ) that only slight differences between  $P E_{F_1}$  for two organisms may result when pronounced differences between  $P E_{F_1}$  occur. These expectations follow from the requirements of the idea that the observed variation is a property of the reacting organism. Hence approximate equivalence of  $P E_{I_1}$ 's at the same values of  $I_m$  for organisms with very different flicker curves cannot be accepted as evidence of common determination through common observational errors introduced by the uniformity of participating observer and procedure in each case.

A consistent conception of the observed relationships can be obtained from the standpoint that the variation encountered is basically a property of the reacting organism. The flicker response experi



ments have the advantage that one can examine  $P E_I$  and  $P E_F$  concurrently in the same organism, and that the critical frequency  $F$ , with the dimensions of a speed, exhibits certain additive properties (Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *d*) which help to justify its interpretation as a measure of the intensity (potential) of the inner driving force responsible for the index reaction, whereas  $I_m$  measures the mean exciting flux required to achieve this inner potential

The relation of  $P E_{I_1}$  to  $I_m$  has been obtained for (1) diverse series of measurements with the same organism and (11) homologous series of measurements with different organisms. Expressed in the same units,  $P E_{I_1}$  for the bee, as a function of  $I_m$ , is lowest in the case of flicker response,<sup>3</sup> 0.3 log unit higher in the visual acuity measurements, and the same when parts of the eyes are opaqued<sup>4</sup>, nearly a full log unit higher in measurements of the time-course of dark adaptation<sup>5</sup>, higher still (as a function of  $I_2$ ) in measurements of intensity discrimination<sup>6</sup>, in the last instance  $P E_{\Delta I}$  is directly proportional to  $\Delta I$ , and is the same fraction of  $\Delta I$  regardless of the widths of the stripes—although decreasing the width of the illuminated bars moves the values of  $I_1$  and of  $I_2$  (where  $\Delta I = I_2 - I_1$ ) to a higher level of intensities. These differences are quite significant statistically, and their order of increasing magnitude is not related to the chronological order of the experiments, the observer was the same person, the responses all involved discrimination of intensities exhibited on alternating stripes of a barred pattern. The order of increasing magnitudes of  $P E_{I_1}$  does, however, correspond to certain differences in type of excitation involved in each experiment.

proportionality factors for rod and for cone branches of the curve are quite different

From such facts it is impossible to conclude that there is a determination of the quantitative dependence of  $PE_{f_1}$  upon  $I_m$  through the activity of the observer. At the same time it is not proved that this is untrue, either

An analogous situation arises from the investigation of temperature characteristics. The general fact encountered is that if heart beat frequencies are measured at various temperatures, or breathing movements, or frequencies of locomotor movements, the same number of determinations (on the basis of the same number of beats) being made at each temperature, then the latitude of variation tends to be about 8 to 10 per cent of the mean and is independent of temperature.<sup>8</sup> (In some cases<sup>9</sup> the latitude of variation may be less, although this depends partly upon the number of beats involved in a count.) There is no possibility of regarding this kind of variation as due to the observer or to the observational method. Yet it is of the same order of magnitude in quite different organisms, in which the absolute frequencies of the events observed differ enormously and for which the temperature characteristics are very unlike, in these respects it corresponds exactly to the properties of  $PE_{f_1}$  as a function of  $I_m$  in the various flicker response curves we have obtained for different organisms under the same conditions. The parallel extends even to the occurrence of differences in the coefficient of variation on either side of a break in the curve connecting mean frequency of activity with independent variable, good cases have been recorded in temperature curves<sup>10</sup> which exactly correspond to changes in the ratio of  $PE_{f_1}$  to  $I_m$  in the composite flicker response curves of fishes, in neither case do these changes in proportionality constant invariably occur, yet the difference in this respect between *Anax* and the fishes is real and striking.

The point has been made in the treatment of temperature characteristic data that the latitude of variation of a frequency for a given activity in the same individual may be the same fraction of the mean

<sup>8</sup> Crozier, 1929, 1935, Crozier and Stier, 1924-25. Crozier and Federighi, 1925. Pincus, 1930-31, Crozier, 1934-35.

<sup>9</sup> Crozier, Pincus, and Renshaw, 1934-35.

<sup>10</sup> Crozier and Stier, 1926-27, Crozier, 1934-35.

frequency independently of conditions other than temperature which modify either the mean frequency at given temperature or the temperature characteristic.<sup>11</sup> This closely parallels the general fact that two flicker response curves may be quite different while only slight differences appear in the plot of  $P E_{I_1}$  as a function of  $I_m$ . On the other hand, our various *Platy* types give essentially the same flicker response curve, and the  $P E_{I_1}$ - $I_m$  curves are indistinguishable, while the two sunfish genera (Table III) give  $F$ - $I_m$  curves which are indistinguishable although for these two fishes the values of  $P E_{I_1}$  differ by a factor of 10.

(With reference to the plot in Fig. 7 it is to be remarked that the difference between, for example, the variation data for *Xiphophorus* and *Platy* seems slight, owing to the scale and the use of logarithmic coordinates. The difference really amounts (for the lines of central tendency) to a consistent factor of 1.06, for others it is larger, if the figures could be adequately presented on an arithmetic grid, their divergence would be obvious. The "second order differences" are thus really considerable.)

The absolute values of  $P E_{I_1}$ , as a function of  $I_m$ , have about the same order of magnitude in very different sensory effects, for example, in measurements of auditory intensity discrimination (Upton and Crozier, 1936), in judgments of differences in lifted weights,<sup>12</sup> and in tests of visual functions with the human eye. Second order differences of the sort already noted occur in these cases also.

Another aspect of this matter is revealed by the interpretation of the Weber fraction  $\Delta I/I_1$  (Crozier, 1935-36, 1936) as a function of  $I$ . From the flicker response curve and the visual acuity test curve for the same organism (the bee), the fraction may be computed on the assumption that  $\Delta I = 1/\sigma_{I_1}$ . The agreement is quantitative despite the fact that the two curves are of different shapes and that

$P E_{I_1}$  as a function of  $I_m$  is distinctly different for the two tests. This result, which also agrees quantitatively with the directly measured values of  $\Delta I/I_1$ , bespeaks an inner coherence of these data due to a property of the reacting animal and is incomprehensible on any other basis.

The point to this review of the general situation is, that the proportionality of  $P E_{I_1}$  to  $I_m$  appears in a great diversity of situations, and that, grossly speaking, the proportionality constant is usually of the same order of magnitude. Closer examination discloses significant differences (second order differences, perhaps, but consistent and not to be ignored). Neither these differences nor the general order of magnitude of the variation is really to be accounted for in a reasonable way by referring it to error of observation. Its general magnitude is determined by some common feature of biological organization, its specific differences reflect specificities in the mechanisms concerned in particular events under scrutiny. It does not in any sense support "indeterminism" (cf. Crozier, 1929, 1935, cf. Cohen, 1936). Unquestionably it also includes error of observation in the classical sense, but the data do not belong in the category of measurements of physical quantities where the numerical observations are subject to a restoring (anti-dispersive) constraint. The separation of these constituents can only be achieved by experimental procedures, of the sort already indicated in studies of the precision of tropistic orientation.<sup>12</sup>

It remains to consider our second point ( $\beta$ ), namely the mechanism whereby a general correspondence may be brought about between  $P E_{I_1}$  and  $I_m$  even in the cases where the  $F I_m$  curves and the values of  $P E_{F_1}$  are very different. This consideration is important for the evaluation of the notion that  $P E_{I_1}$  may be merely observational error and not an intrinsic property of the tested organism.

Take first the flicker response curves for *Anax* and *Enneacanthus* (Crozier, Wolf, and Zerrahn Wolf, 1935-36 a, b). The discussion might with equal propriety have been begun by dealing with the curves of mean critical flicker frequency as a function of intensity, rather than (as we have done) with the  $F I_m$  data. If we really have to do with error of manipulation, then (since the technic employed in the 2 cases was identical),  $P E_{F_1}$  should be the same at identical values

<sup>12</sup> Crozier and Pincus 1929-30, 1, b

of  $F_m$  for the two organisms. Yet the  $PE_{F_1}$  for the sunfish is uniformly about 100 per cent greater. Despite this difference, if from the width of the band formed by  $F_m \pm PE_{F_1}$  as a function of  $I$  one calculates the expected magnitudes of  $PE_{F_1}$  as a function of  $I$ , it turns out that the two sets agree in the manner and to the extent which we have already noticed in the directly determined quantities. (The reverse calculation is discussed in Crozier, 1935-36, Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *a, b*.) One cannot attribute both the unlikeness of the  $PE_{F_1}$  series and the similarity of the  $PE_{F_1}$  data to common experimental error in each series, on the contrary, although including this factor, they are fundamentally both determined by a property of the reacting organism, this property, expressed as a variability of performance under constant conditions, carries with it the necessity for fluctuation in the potential of an exciting energy required to effect a given amount or extent of performance.

The theory of response to flicker which we have considered regards marginal recognition of flicker as a phenomenon of intensity discrimination. The effects discriminated are (1) that due to the (average) action of a flash of light, and (2) that represented by the after-action (after-image) persisting during the intervals of no light (Crozier, 1935-36, Crozier, Wolf, and Zerrahn-Wolf, 1935-36 *c, d*). We may suppose that recognition of flicker requires the establishment

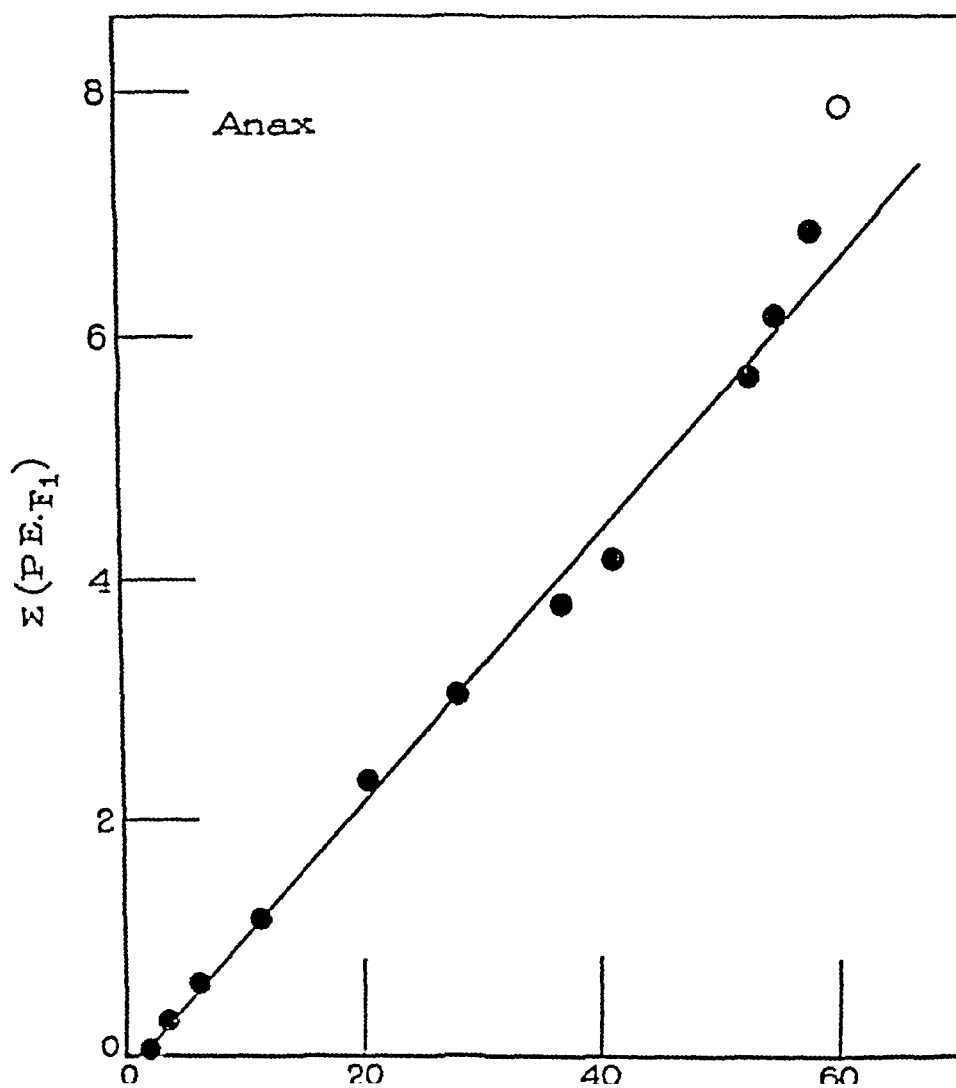
tion in effect, will be thus given by  $dN/d\log I$ , where  $N$  is the total number of excitable elements, and will pass through a maximum as  $\log I$  increases. This corresponds with the properties of  $P E_{F_1}$ , because, at fixed  $I$ ,  $P E_{F_1}$  measures the fluctuation in the effect of a flash ( $\epsilon$ , the action of the intensity  $\times$  the duration of the flash is constant for a given end result). With (at any moment) a slightly larger number of excited contributing elements, the duration of a flash of given intensity need not be quite so long to produce the same magnitude of effect and therefore the same intensity decay curve in the after image, and so the same difference between the two. On this basis the integration of  $P E_{F_1}$  over the whole range of intensities should give a proportionate measure of the total number of excitable elements. The integration is made graphically, from the curves drawn through the measurements of  $P E_{F_1}$  as a function of  $\log I$  (in Crozier, Wolf, and Zerrahn Wolf, 1935-36 *a, b*).  $\Sigma P E_{F_1}$  as a function of  $\log I$  should then be parallel to  $F$  as a function of  $\log I$ . The simplest method of demonstrating the correctness of this expectation is obtained by plotting  $\Sigma P E_{F_1}$  against  $I$ . For the *Anax* data the result is exhibited in Fig. 8. The rectilinear relationship is striking, the deviation of the last point ( $F_{max}$ ) is due to instrumental error at the highest intensity used, as with the case of the sunfish (Crozier, Wolf, and Zerrahn Wolf, 1935-36 *a, b*). The data for *Enneacanthus*, treated in the same manner, are given in Fig. 9. As already implied from other relationships, the proportionality constant should differ for rods and cones. The break in the relationship comes exactly at the point identified as that at which the rod contribution fades out of the picture (Crozier, Wolf, and Zerrahn Wolf, 1935-36 *d*). This empirically verified conception is therefore embodied in the expression

$$P E_{F_1} = k_1 dF/d\log I = k_2 IdF/dI \quad (I)$$

(When  $dF/dI = 0$ , at  $F_{max}$ , the variation encountered is of another sort, as already indicated.)

With fixed  $F$  and an all or nothing index of response, the presumed condition for response (other factors constant) being a certain percentage difference between sensory effects due to light flash and to after image, then  $I_1$  must vary from time to time in such a manner that

$$P E_{I_1} = k P E_{F_1} dI/dF \quad (II)$$



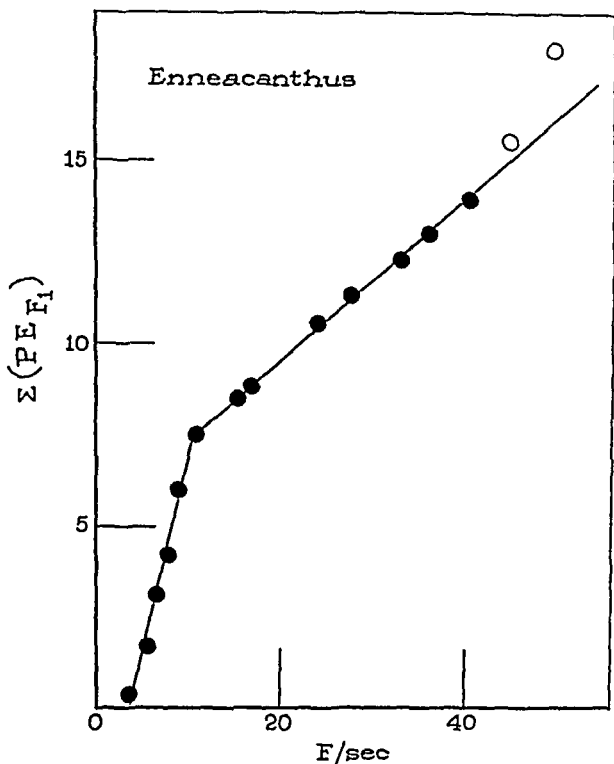


FIG 9 Similar to Fig 8, but based upon observations with the sunfish *Enneacanthus* (Crozier, Wolf, and Zerrahn Wolf, 1935-36 a). The abrupt break coincides precisely with the dropping out of the rod contribution to the determination of critical flicker frequency as determined by independent criteria (cf Crozier, Wolf, and Zerrahn Wolf, 1935-36 a d). In each zone, respectively of predominant rod function and cone function,  $d(\Sigma[P E F_i])/dF$  is constant.



since  $P E_{F_1}$  measures the spontaneous fluctuation of excitability at fixed  $I$  and  $dI/dF$  is the mean equivalent change of  $I$  per small change of  $F$ , the equation is thus dimensionally balanced

From these 2 equations,

$$P E_{I_1} = C \frac{(dF)(dI)(I)}{(dI)(dF)} = CI \quad (III)$$

This is the relation of  $P E_{I_1}$  to  $I_m$  which has been obtained experimentally. The proportionality constant  $C$  contains no reference to the flicker frequency or to the shape of the  $F$ - $I$  curve. Hence  $P E_{I_1}$ , as a function of  $I$ , should be independent of  $F$ . We find empirically that the constant  $C$  has the same general order of magnitude for different organisms, although their  $F$ - $I$  curves may differ profoundly.

This can be approached in another manner, more general, by a procedure for which we are indebted to Dr. Charles P. Winsor. We have a situation in which the observed result (response to flicker) is conceived to be a function of three variables,  $F$ ,  $I$ , and  $Z$ , where  $F$  and  $I$  are measured and  $Z$  is a random variable (due to the organism).

From (4), (5), and (6)

$$\frac{\sigma_F}{\sigma_I} = \frac{\delta F}{\delta I} \quad (7)$$

which is the result already obtained. It therefore follows that given experiments in which  $F$  or  $I$  is the independent variable, we should be able to predict the scatter of the dependent variable when the experiment is performed with the reverse arrangement.

If in addition it is to be assumed that the recorded measurements of  $F$  and  $I$  are subject to scatter independent of  $Z$ , in other words to experimental error in the customary sense, then

$$F = \phi(I, Z) + \epsilon \quad (8)$$

$$I = \psi(F, Z) + \eta \quad (9)$$

These give

$$\sigma_F^2 = \left( \frac{\delta \phi[F, \bar{Z}]}{\delta Z} \right)^2 \sigma_Z^2 + \sigma_\epsilon^2 \quad (10)$$

$$\sigma_I^2 = \left( \frac{\delta \psi[I, \bar{Z}]}{\delta Z} \right)^2 \sigma_Z^2 + \sigma_\eta^2 \quad (11)$$

and

$$\frac{\sigma_F^2 - \sigma_\epsilon^2}{\sigma_I^2 - \sigma_\eta^2} = \left( \frac{\delta F}{\delta I} \right)^2 \quad (12)$$

This is analogous to (7) but no *a priori* assumptions can be made as to  $\sigma_\epsilon$  and  $\sigma_\eta$ , hence the calculation of  $\sigma_F$  from  $\sigma_I$  or the reverse cannot be undertaken. At the same time if (7) is adequate it follows that the  $\epsilon, \eta$  terms do not enter significantly, this amounts to the conclusion that the variation dealt with is practically free from experimental error in the usual sense. This is entirely consistent with the fact that duplicate determinations of  $I_m$  agree much more closely than their  $\sigma_I$ s would warrant (save in the exceptional and specifically accounted for case of the threshold for cones in *Alphaphorus*, already discussed).

We are therefore in position to conclude that a consistent account of the characteristics and the interrelationships of the variabilities of our measurements can be given from the standpoint that this variability is a property of the reacting organism.

## VI

### *Black Hellert Hybrids*

Our desire has been, in part, to prepare ground for a genetic analysis of differences such as the curves in Fig. 1 reveal, and which can be

rather simply characterized by descriptive constants assembled in Table IV. The desirability and the importance of such analysis derives from two sources, which from the standpoint of logical experimentalism may be regarded as distinct. (1) it is the necessary procedure for the characterization of the really significant differences, namely *functional* differences, with which genetic theory must ultimately deal, it implies functional formulation, and the genetic properties of such formulations require to be investigated, and (2) it provides a relatively simple and efficient means of deciding whether constants in quantitative formulations have a definite significance, a reality apart from the descriptive efficacy given by curve fitting, and thus may be hopefully regarded from the viewpoint of theoretical interpretation as to mechanism. The complexity of a biological phenomenon, since its properties are specifically reproducible, must be subject to and governed by a determinate control. The complexity increases the labor of experimental inquiry and the difficulties of interpretation, but the possibility of genetic manipulation presents a unique and counterbalancing advantage.

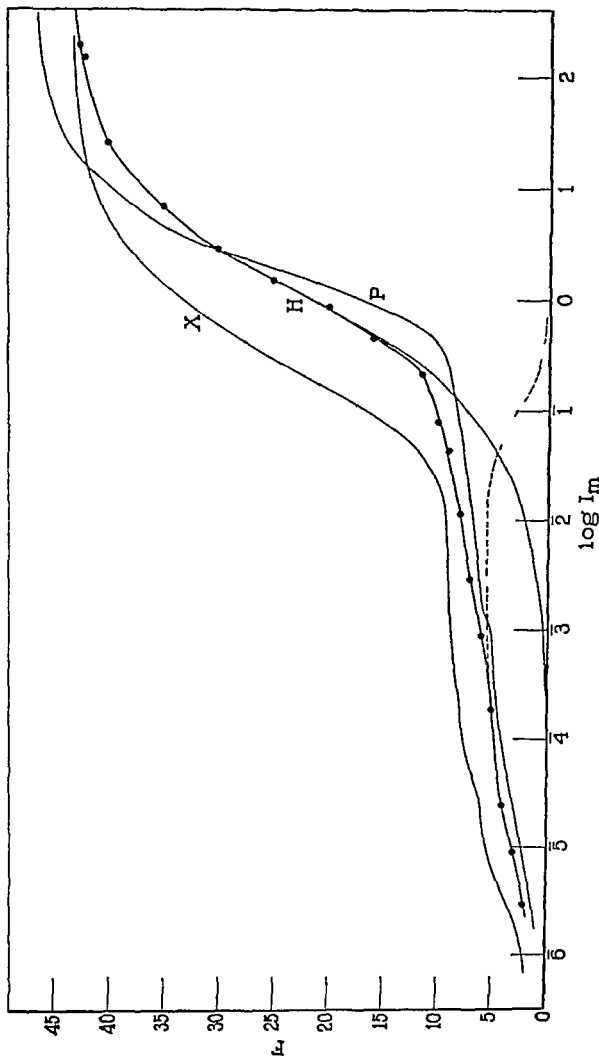


Fig 10 Mean critical illumination  $I_m$  as a function of  $F$  for a homogeneous group of backcross hybrids between swordtail (X) and Platy (P). See text. The curves for X and P are transferred from Fig 1. The cone segment logistic (Fig 8) is extrapolated to give the presumptive level of intensity for the entrance of activity due to cone excitation. The difference curve has been plotted as in Fig 5.

The values of  $I_m$  and of  $P E_{I_1}$  for the hybrid stock are given in Table IV,  $I_m$  as a function of  $F$  is plotted in Fig 10. A certain re-

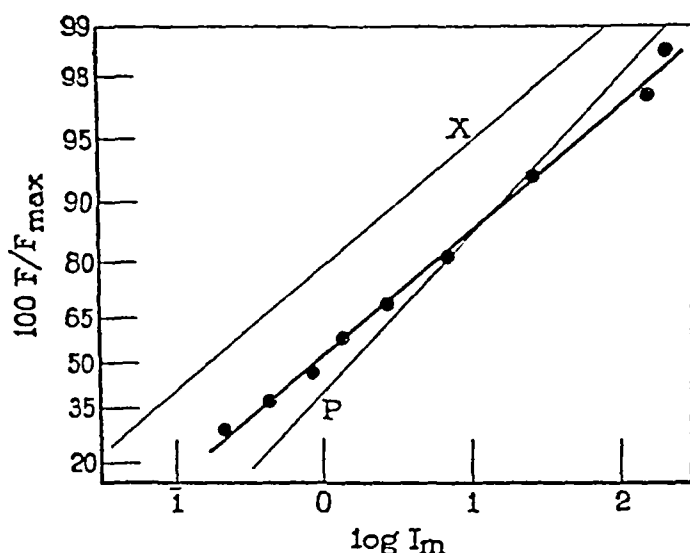
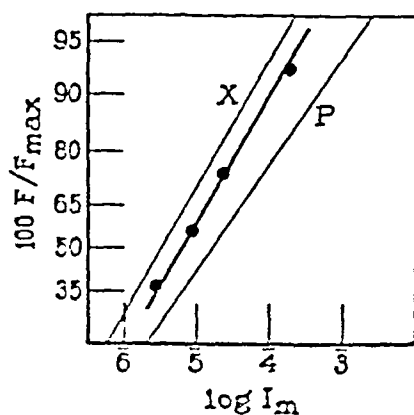
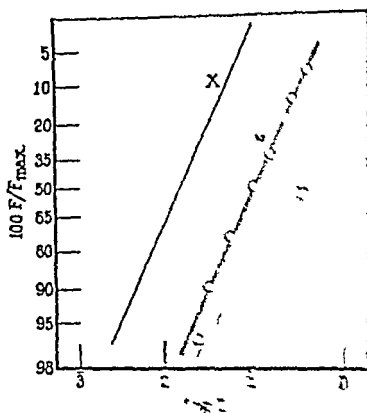


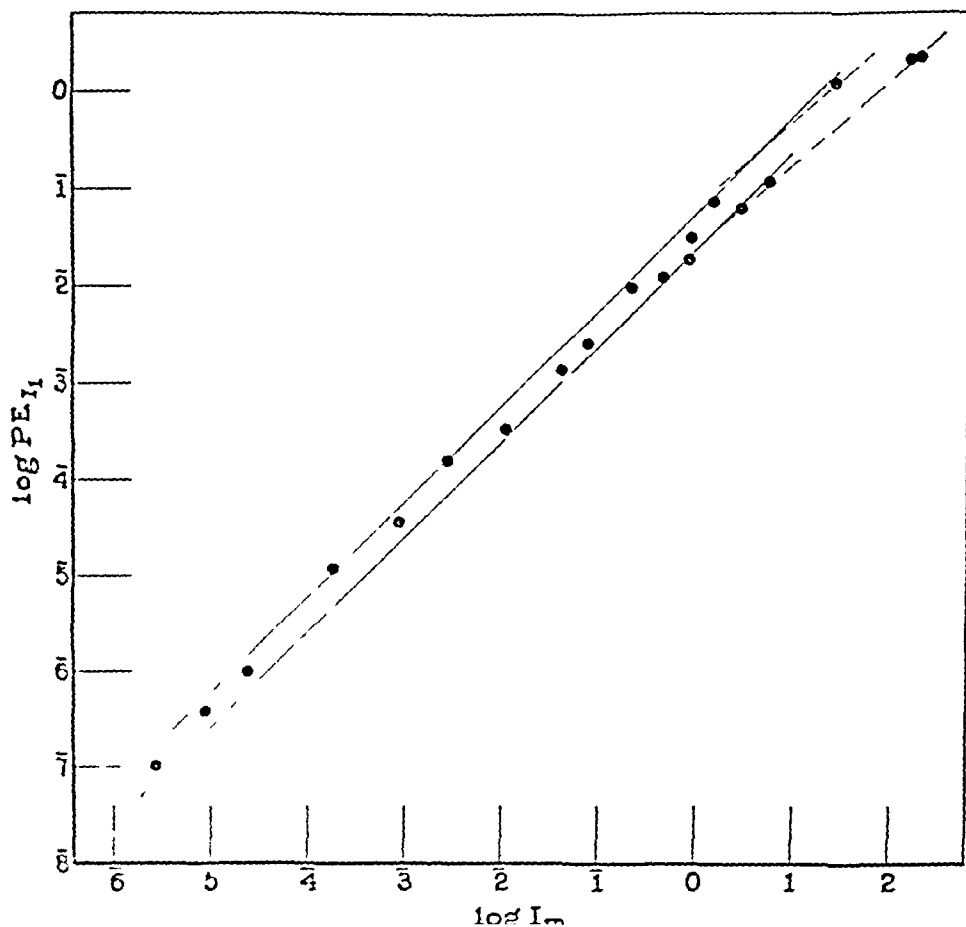
FIG 11 The cone segment of the flicker response curve for the Black Hellert hybrids gives the same logistic exponent,  $p = 1.90$ , as does the cone curve for the swordtail parental stock.



position rather similar to that occupied by the graph for the *Platyacilius* ancestor. These considerations can be made more definite by the examination of the constants in the descriptive equations for the respective flicker curves (Table V). Comparisons are possible because the logistic also describes the data for the hybrids (Figs. 11 and 12). With our apparatus it was not possible to obtain accurate settings of intensity at higher intensities than antilog 2.5 millilamberts, we were



obtained as before by difference on the basis of the extrapolated cone logistic, has also the exponent characteristic of the declining curve for *Xiphophorus* rods (Fig 13) This adds to the evidence for lack of influence of the cone function upon the declining contribution of



the rod  $I_{max}$ . The difference between the *Lepophorus* and hybrid curves is not similar to the change produced by alteration of temperature (Crozier, Wolf, and Zerrahn Wolf, 1935-36 d). The values of  $I_1$  for rods and cones, the intensities for  $I = 0.5 I_{max}$ , are not equally affected in the new combination, so that the mode of junction of the rod and cone curves has a different aspect. The extrapolation of the logistic for the cone curve, however, approximates  $I = 0$  at precisely the right level of intensity for correlation with the bump on the composite curve (Fig. 10).

The variability of  $I_1$  is exhibited in Fig. 14, there is no distinct break in the plot of  $PE_{I_1}$  vs  $I_m$ , although it is possible that measurements at still higher intensities would show something of the sort. The relation between  $PE_{I_1}$  and  $I_m$  is described by

$$(1 \pm 0.402)PE_{I_1} = 0.374 I_m$$

The proportionality constant is just the mean of those for swordtails and Platys (see section V), the spread coefficient of  $PE_{I_1}$  is lower than for either of the parental stocks.

## VII

Comparison of the flicker response curves for the four fishes thus far studied in some detail permits conclusions relative to certain points in the theory of visually excited responses, which may be commented upon briefly.

Differences between these curves as obtained at one temperature involve not merely shifts in position on the intensity axis, but also changes in scale and in the proportions of constituent parts. The same type of quantitative formulation is, however, applicable to all of them. Rod and cone activity constituents derivable from two parent stocks serve to synthesize the result obtained in a hybrid stock produced from these parent lines.

The "elements of action" involved in controlling the responses must be presumed to be of central nervous location. These elements are clearly correlated, however, with discrete retinal properties. Of these the most conspicuous is the separability of rod and cone functions as required by the duplicity doctrine (Kohlrausch, 1922, von Kries, 1929, Hecht, 1934). For convenience we may speak of rod thresholds,



populations of rod and cone effects, and the like, without its being assumed or implied that properties of the flicker curves are directly representative of the excitabilities of the corresponding retinal elements. With this reservation as to the use of the terms rods and cones, we note that in our group of flicker response curves (i) higher rod threshold does not necessarily mean a smaller population of rod effects, (ii) or a more rapidly rising rod curve, (iii) or a higher cone threshold, (iv) or a steeper cone curve, and that (v) there is no correlation between the steepness ( $p$ ) of a cone curve and its maximum critical flicker frequency, or (vi) between  $p$  and general intensity level. Threshold,  $F_{max}$ ,  $p$ , and  $I$ , are separately and independently determined.

This body of evidence, coupled with the analyses of the individual flicker response curves, supplies a proof of a new kind that the basic requirement of the duplicity theory is correct: the retinal functions of vertebrates involve the distinct operations of two groups of elements, rods and cones.

## VIII

### SUMMARY

Flicker response curves have been obtained at 21.5°C for three genera of fresh water teleosts: *Enneacanthus* (sunfish), *Xiphophorus* (swordtail), *Platyphacchilus* (Platy), by the determination of mean critical intensities for response at fixed flicker frequencies, and for a certain homogeneous group of backcross hybrids of swordtail  $\times$  Platy (Black Hellen).

The curves exhibit marked differences in form and proportions. The same type of analysis is applicable to each, however. A low intensity rod governed section has added to it a more extensive cone portion. Each part is accurately described by the equation

$$I = F_{max} / (1 + e^{-p \log I/I_0}),$$

The hybrid Black Hellens show quantitative agreement with the *Xiphophorus* parental stock in the values of  $p$  for rods and cones, and in the cone  $I_m$ , the rod  $I_{max}$  is very similar to that for the Platy stock, the general level of effective intensities is rather like that of the Platy form. This provides, among other things, a new kind of support for the duplicity doctrine. Various races of *Platyphocilius maculatus*, and *P. variatus*, give closely agreeing values of  $I_m$  at different flicker frequencies, and two species of sunfish also agree. The effect of cross breeding is thus not a superficial thing. It indicates the possibility of further genetic investigation.

The variability of the critical intensity for response to flicker follows the rules previously found to hold for other forms. The variation is the expression of a property of the tested organism. It is shown that, on the assumption of a frequency distribution of receptor element thresholds as a function of  $\log I$ , with fluctuation in the excitabilities of the marginally excited elements, it is to be expected that the dispersion of critical flicker frequencies in repeated measurements will pass through a maximum as  $\log I$  is increased, whereas the dispersion of critical intensities will be proportional to  $I_m$ , and that the proportionality factor in the case of different organisms bears no relation to the form or position of the respective curves relating mean critical intensity to flicker frequency. These deductions agree with the experimental findings.

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# THE EFFECT OF HYDROGEN ION CONCENTRATION UPON THE INDUCTION OF POLARITY IN FUCUS EGGS

## II THE EFFECT OF DIFFUSION GRADIENTS BROUGHT ABOUT BY EGGS IN CAPILLARY TUBES\*

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### INTRODUCTION

It has been known for a long time (Rosenvinge, Kniep, Hurd, and Whitaker (1-4)) that *Fucus* eggs in the dark tend to form rhizoids in the resultant direction of neighboring eggs. It was shown by Whitaker (4) that unfertilized eggs of another species act as directing agencies, and therefore the effect is non specific and is not dependent upon growth activity in the directing cells.

More recently (Whitaker (5)) it has been shown that in *F. furcatus*, when eggs are reared in the dark at  $14\frac{3}{4}^{\circ}\text{C}$  in normal sea water at pH 7.8-8.0, aggregations of at least ten or more eggs in close proximity are necessary in order to bring about appreciable mutual control of the polarity. Groups of five eggs or two eggs alone in a dish do not show the effect. These relations suggested that a greater concentration of substances diffusing from the eggs than can be provided by the smaller groups of eggs under conditions of free diffusion is necessary to cause the eggs to respond to each other. Since  $\text{CO}_2$ , and presumably other acid metabolites are included among the diffusion products, aggregations of various sizes were reared in sea water acidified to pH 6.0. In this medium, all aggregations, including groups of only two eggs alone in a dish, now showed strong mutual inductions of polarity. It was concluded that increased hydrogen ion concentration augments or intensifies the mutual inductive effect (5).

\* This work has been supported in part by funds granted by The Rockefeller Foundation.

*Purpose*

In the present experiments, single eggs have been reared in the dark within and near one end of a capillary tube so that diffusion products escape more rapidly from one side of the egg than from the other. Also, due to the presence of the tube wall, which limits diffusion, the equilibrium concentration of diffusion products (and also oxygen lack) immediately about the egg is presumably considerably greater than about an egg or a small group of eggs under conditions of unrestrained diffusion. The purpose of the experiments has been to see whether the polarity of an egg under such circumstances might be determined by the gradients resulting from its own metabolism, and to find the effect of initial pH of the medium on the results. Another and original purpose was to test the feasibility of culturing single eggs in capillaries in order to apply gradients of known substances across them, but it is first necessary to know the response of an egg in a capillary to the gradients which the egg itself produces when the medium is initially homogeneous throughout.

After the discovery of the striking effect of the pH of the medium on the intensity of the neighbor effect (see Introduction), it appeared quite possible that the response of an egg in a tube to the gradient of its own diffusion products might also be highly sensitive to the pH of the sea water. Subsequent measurements with a glass electrode showed that the pH of sea water collected and filtered as it had been during these earlier experiments ranged over a considerable time from 8.3 to 7.6. More attention to prompt filtering very soon after collecting lessens this range. A new series of experiments was carried out in January, February, and March, 1936 with measurement and artificial control of the pH of the sea water. These new experiments are the basis of the following report.

*Making and Preparing the Capillary Tubes*—Clean new pyrex tubing was drawn out to form capillaries which were selected for internal diameter as measured by ocular micrometer. By means of a Taylor micromanipulator pieces of suitable bore were cut quite squarely into measured lengths between a diamond splint and the edge of a razor blade. The capillary lengths used were 400, 700, and 1400 or 1500 microns. As the eggs vary in diameter from about 65 to 90 microns, averaging about 75, these lengths correspond approximately, on the average, to 5, 10, and 20 egg diameters. Large numbers of capillaries were cut, and by selecting eggs and capillaries of appropriate diameters, any degree of clearance between the egg and the capillary could be obtained. Similar capillaries were also made from clear fused quartz (silica).

To reduce dangers of contamination of various sorts, capillaries were never used a second time. The new tubes were placed one each in a small Syracuse dish (Bureau of Plant Industry model) in 1 cc sea water. A fine glass pipette which was held in the hand and operated by a rubber tube to the mouth was placed against one end of the short capillary on the bottom of the dish and a large volume of medium was sucked through to flush it. After being flushed and then soaked overnight (usually 24 hours) in sea water, each capillary was transferred through three dishes of the medium to be used in the experiment and was copiously flushed in each to remove any solutes from the glass walls of the capillary and to equilibrate with the experimental medium.

*Placing the Eggs*—Eggs were fertilized in normal sea water and then between 30 and 90 minutes after fertilization (during which time the cell wall progressively becomes more adhesive) a single fertilized egg was placed in each small Syracuse dish of experimental medium containing a prepared capillary. Eight of these dishes had already been placed in a levelled Petri dish on the stage of a microscope. The Petri dish also contained sea water and served as a moist chamber. Several microscopes each bearing such a levelled Petri dish, were mounted on a heavy concrete table in the constant temperature room as it was found that either vibration or slight slope of the capillaries would cause the eggs to move along the capillaries or to roll out. The Petri dish was not moved from the stage of the microscope after the eggs were placed.

Under the compound microscope an egg was washed and placed near one end of the capillary and medium was gently sucked through the capillary from the

other end by means of the mouth pipette. This carried the egg into the capillary without abuse or distortion and the distance from the end of the capillary at which the egg came to rest could be accurately regulated. If the egg came too far into the capillary it was rolled back by gently blowing medium into the opposite end of the tube. The stickiness of the egg at this time reduced the tendency to move. By about 2 hours after fertilization the sticky wall has hardened and attaches the egg to the glass. After an egg was placed in each of the eight capillaries in a moist chamber, the dishes containing the capillaries were rotated so that the capillaries lay in different positions with respect to the compass as a control against any unknown directional influence in the environment. The compass direction of the capillary had no effect on the result. The results were observed and either drawn or photographed about 24 hours after fertilization when the rhizoid protuberance was well developed and the spore had usually developed to the two cell stage.

*The Experimental Medium*—The medium inside and outside the capillary was always the same at the start of the experiment. Eggs were reared in normal sea water of measured pH, ranging in these experiments from 8.09 to 7.85 (see Table I). In the same experiment, eggs were also reared in samples of the same sea water which had been artificially made more acid or more basic. In some cases the sea water was acidified by adding from 3 to 5 volumes to the hundred of acid McIlvaine's secondary sodium phosphate-citric acid buffer. In most cases concentrated HCL instead was added to the sea water in such small amount as to have no appreciable effect on the total osmotic pressure. Sea water was artificially made more basic by adding from 1 to 5 volumes to the hundred of a basic isosmotic mixture of  $\text{Na}_2\text{CO}_3$  and  $\text{NaHCO}_3$ .

After the addition of the HCL or the  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  mixture, the sea water

medium in the small dish was measured. The changes which had occurred during the progress of the experiments are shown in column 8 of Table I.

### RESULTS

In more than 600 cases studied, the rhizoid protuberance formed either on the side of the egg toward the far end of the capillary into which it extended, or else it formed on the opposite side of the egg *i.e.* on the side toward the near end of the capillary in which case it extended toward or out of this end of the capillary. Photomicrographs in Fig. 1 show eggs in each category located at several different distances from the end of the capillary. Table I gives the conditions of the experiments and the number of eggs in each experiment. The percentage of eggs in the different media of each experiment which formed the rhizoid protuberance on the side of the egg toward the far end of the capillary *i.e.* on the side of the egg from which diffusible substances from the egg could diffuse away least readily is given in column 10 of Table I.

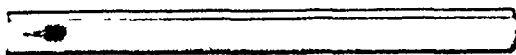
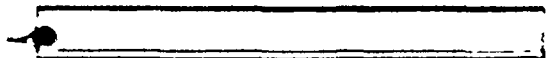
These percentages bear a very definite relation to the initial pH of the medium (*i.e.* the pH at the start of the experiments as shown in Table I, column 7) which remained essentially unaltered outside the capillary during the course of the experiments. The percentages are plotted against initial pH in Fig. 3 which shows that in acid sea water a very high percentage of the rhizoid protuberances form toward the far end of the capillary. Near pH 8.0, however, the percentage drops rapidly to 50, which represents a condition of random direction, and at higher pH values up to 9.5 or 9.6 the percentage drops down to less than 25. In other words in basic sea water most of the rhizoid protuberances form on the opposite side of the egg toward the near end of the capillary. As the sea water is made more basic, the percentage of rhizoid protuberances forming toward the near end of the tube does not approach 100 however as in the reverse phenomenon in acid sea water, but attains only 75 or 80 at the maximum.

In order to find out if this percentage would more nearly approach 100 under still more basic conditions 59 eggs were tested at pH 9.1–9.2. A complication comes in, however, since above about pH 8.6 precipitation of certain salts from the sea water takes place and it is not known what effect this may have on the development of polarity.





A



B



A



B



C

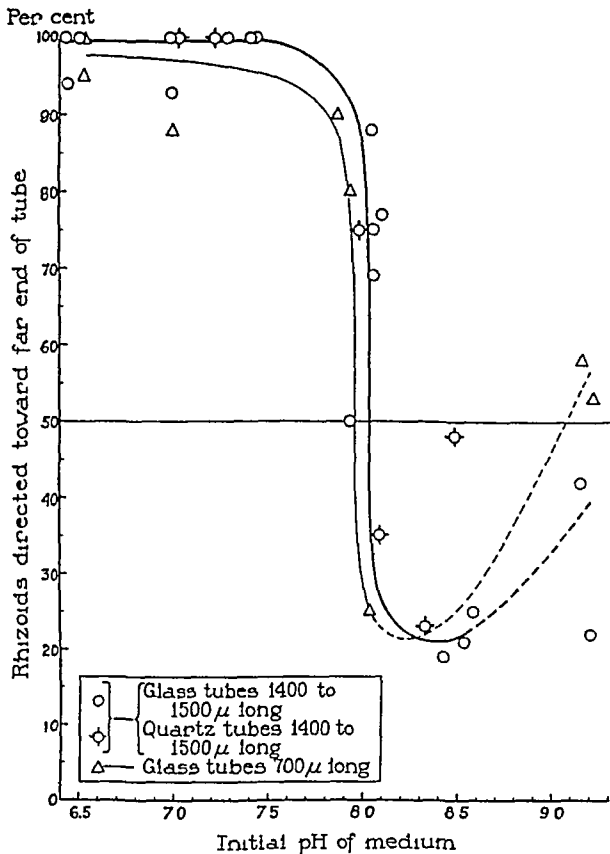


FIG 3 Graph showing percentage of eggs which formed rhizoids on the sides toward the far ends of the tubes (where diffusion is most impaired) when the pH of the medium initially (*i.e.*, when the eggs were placed in the tubes at the beginning of the experiments) was as shown. The curves are freely drawn. In the more basic range (curves intermittent) special considerations make the results less certain (see text). The response of the eggs to their own diffusion gradients is seen to be somewhat less pronounced in the shorter tubes as might be expected. The significant data upon which each point is based are shown in Table I.

TABLE I

Conditions of the experiments, and results upon which Fig 3 is based. The columns, left to right, show (1) number of the experiment, (2) capillary tube lengths to nearest 100 $\mu$  (tubes are pyrex in Experiments 1 to 8 inclusive, and quartz in Experiments 9 and 10), (3) clearance between egg in tube and tube wall (range and average), (4) distance from near end of tube to nearest edge of egg (range and average), (5) composition of medium, which was either unaltered sea water, or sea water to which acid or base was added (see text), (6) pH of medium at end of adjustment period (see text), (7) pH at start of experiment, when eggs were placed in tubes, (8) change in pH of medium surrounding tubes as measured 24 hours later, (9) number of eggs, each in a separate tube and dish, (10) per cent of these which formed rhizoids on sides toward far ends of tubes. See text.

1	2	3	4	5	6	7	8	9	10
Exp. No.	Tube length in $\mu$	Average C. change in pH in 24 hrs in $\mu$	Average C. change in pH in 24 hrs in $\mu$	Medium	pH at end adjustment period	pH at start of experi- ment	pH change 24 hrs after start of ex- periment	No. of eggs	Rhizoids toward far end of tube

in the egg. There is a marked difference in the type of the rhizoid protuberance which forms at such high pH (Fig 2). It is decidedly narrower at the base and its extension is retarded. There is no indication whether this is a result of the high pH as such or whether it is due to the loss of precipitated salts from the sea water. At this high pH the eggs in the capillaries show a reduction of the tendency for rhizoid protuberances to form toward the near end. The percentage drifts back toward 50, or randomness of the results. The number of experiments is less in the extreme basic range, and the special circumstances of precipitation and altered developmental type combine to make this apparent reduction in the control of polarity difficult to interpret at the present time. The results here must be regarded as merely exploratory and tentative. However, it does appear relatively safe to conclude that as the pH is increased from 8.5 to 9.2 the percentage of the protuberances forming toward the far end of the capillary does not approach 0. The effect of the base in controlling the response of the egg does not become almost absolute, as in the case of the acid.

In addition to the experiments recorded in Table I and Fig 3, 50 eggs were reared in four experiments which were similar to the others except that the glass capillaries were only 400 microns long. This is only slightly over 5 egg diameters, and eggs which were 1 egg diameter or more in from the end of the capillary were therefore fairly close to the center of the capillary. In consequence, the difference in diffusion from the two sides of the egg was less than in the case of longer capillaries. The results were very similar to those obtained in the longer capillaries, but as might be expected, the determinative effects on the eggs were somewhat less marked. In Table I and Fig 3 it may also be seen that the response of the eggs in acid sea water to the gradients in the capillaries was not quite as strong in capillaries 700 microns long as in capillaries 1400 or 1500 microns long.

#### DISCUSSION

When a single *Fucus* egg develops in a gradient of its own diffusion products in a medium which is initially homogeneous, the developmental response of the egg to the gradient appears to be greatly affected by the hydrogen ion concentration of the medium. It has

already been shown (Whitaker (5)) that when two eggs develop near each other, the rhizoid protuberances develop on the side toward the neighbor if the sea water is acidified, while in normal sea water they do not. The present results are quite in agreement with this observation and a reasonable interpretation is that upon sensitization with a slight amount of acid the rhizoid protuberance develops on the side of the egg subjected to the greatest concentration of diffusion products of the egg itself for the same reason that it develops on the side toward a neighboring egg.

In the case of the egg in a capillary, a lesser acidification of the medium suffices to sensitize the egg, so much less than sea water as collected is often sufficiently acid without alteration. This is not surprising because a consideration of diffusion areas shows that an egg in the end of a capillary is subjected to a greater concentration of diffusion products of the egg (including  $\text{CO}_2$ ) than are two eggs in a dish under conditions of unrestricted diffusion. The developing *Fucus* egg has a relatively high respiratory rate in the dark. It is of the order of 5 times that of a fertilized sea urchin egg (Whitaker (7-8)). The single egg in the capillary therefore undoubtedly increases acidity about itself very considerably, particularly on the side with more restricted diffusion.

can be given only as an observation. Extended discussion of this phenomenon would be premature at the present time. It might be noted, however, that although the relation was not stressed in a previous paper, the data in Table I (Whitaker (5)) show that two eggs alone in a dish in the most basic medium used (pH 7.8-8.0) showed a statistical tendency to form rhizoids on the sides of the eggs away from the neighbor.

The variable results obtained in capillaries in 1934 (see page 58) can be explained adequately by the variations of pH of the different samples of sea water as it was collected at that time (see Fig. 3).

In the case of the single egg in the capillary, responding to its own diffusion gradient, in contrast to the aggregations of two or more eggs affecting each other, there appears to be no possibility that the effect is mitogenetic.

*Errors and Alternative Interpretations*—So far in the discussion the view has been favored that the polarity of the egg is determined, depending on the hydrogen ion concentration of the medium, by a gradient of products diffusing from the egg. Two or three other possibilities must be considered. In the first place, substances coming into solution from the pyrex capillaries would be present in a concentration gradient across the egg. It was with this in mind that only new, clean, soaked pyrex was used and that capillaries of pure fused silica (quartz) were substituted in Experiments 9 and 10, with essentially similar results. If there is any such effect from glass, or an effective differential adsorption from the medium, it also takes place in quartz. It does not appear to the authors very probable that the principal results are due to such factors.

Since the eggs in the dark consume oxygen at a relatively high rate (Whitaker (7-8)) a gradient of oxygen tension must be present across the egg in the capillary, in the opposite direction to the gradient of products diffusing from the egg. A gradient of oxygen tension has never been established across *Fucus* eggs to test its effect. Winkler (10) found that an oxygen gradient across the eggs of *Cystosira*, another member of the Fucaceae, had no effect on the polarity in that form. It has already been noted that a gradient of hydrogen ions and CO<sub>2</sub> does determine the polarity of the *Fucus* egg, at least in slightly acidified sea water, and this together with the pronounced

sensitivity of the phenomenon to pH and the general physiological effectiveness of hydrogen ions and  $\text{CO}_2$ , suggests the greater importance of these substances in the present instance. The rôle of the oxygen gradient has not been determined, however, and it may be an important part of the picture. In any event, it is not supposed that only one substance or its reactions determines polarity. It is much more probable that a number of substances are involved, any one of which might become limiting. The participation of some type of growth substance inside the cell, if not diffusing from it, appears quite probable on general grounds.

When acid is added to sea water,  $\text{CO}_2$  is produced, and when base is added,  $\text{CO}_2$  is absorbed from the air. Until equilibration with the atmosphere has taken place,  $\text{CO}_2$  will continue to be given to or taken from the air for a long time, with a resulting drift of the pH of the sea water. If acidified sea water contained  $\text{CO}_2$  considerably in excess of atmospheric tension at the start of an experiment in a capillary, further diffusion of excess  $\text{CO}_2$  out into the air would proceed more rapidly from the medium outside the capillary than from the medium in the capillary, from which diffusion is partly restricted. This would result in a gradient of  $\text{CO}_2$  across the egg, and would correlate with a rising pH of the medium outside the capillary. Conversely, alkalinized sea water not in equilibrium with atmospheric  $\text{CO}_2$  would produce a gradient of  $\text{CO}_2$  across the egg in the opposite direction. Since such artificial gradients would be in the right direction to explain the results, their magnitude must be considered. The best measure is the drift of the pH of the medium exposed to air.

A comparison of columns 7 and 6 in Table I shows that in 15 of the 18 measurements in Experiments 4 to 10 inclusive, in which the media stood for 13 to 40 hours after adjustment of the pH and before the beginning of an experiment, the change in pH as measured was not more than 0.02 unit. In two cases it was 0.13 unit. All changes which took place were slightly in the acid direction, as might be expected from the action of microorganisms. If acidified sea water had been giving off  $\text{CO}_2$ , it would have become more basic. Column 8 shows the changes in pH of the media surrounding the capillaries in 24 hours after the start of the experiments. Since the place of rhizoid origin is determined in about the first half of this time, only

about half of the changes shown in column 8 took place during the significant period. Column 8 shows that the changes in pH are very slight in most of the experiments, and ordinarily the drift of the pH of the media to which acid or base had been added was no greater than that of the normal sea water. In all of these, the slight drifts are predominantly in the acid direction, even in the acidified sea water. The pH values were measured to 0.01 unit, which did not exceed the limit of the glass electrode. Such highly refined measurement is not regarded as fully significant, but the values are recorded as measured because the significance at least approaches such limits.

In view of the comparative constancy of the pH of the media in most of the experiments, it appears very probable that artificial  $\text{CO}_2$  gradients resulting from delayed equilibrations with the atmosphere after addition of acid or base must have been small compared with the gradients of catabolic  $\text{CO}_2$  established by these rapidly respiring eggs.

#### SUMMARY

- 1 When a *Fucus* egg develops near one end in a close fitting capillary tube of pyrex glass or silica (quartz), diffusion of substances passing to and from the egg is more impeded on the side of the egg toward the far end of the tube.

- 2 The egg therefore develops in a gradient of its own diffusion products, and of oxygen tension.

- 3 More than 600 eggs have been reared, each near one end in a capillary, in sea water at various regulated and measured pH values.

- 4 When the medium, which is initially homogeneous inside and outside the capillary, is initially at pH 6.5 to 7.6, nearly all of the eggs develop rhizoid protuberances on the sides of the eggs toward the far ends of the capillaries. This is on the sides of the eggs where the concentration of substances diffusing from the eggs is greatest.

- 5 The polarity and developmental pattern of the egg is thus determined either by a concentration gradient of products diffusing from it, or by a gradient of oxygen tension. The former interpretation is favored.

- 6 This is regarded as an extension of earlier observations that rhizoid protuberances form on the sides of two neighboring eggs in the direction of the neighbor if the sea water is acidified.



7 It appears hardly possible that a mitogenetic effect could be responsible for the response of an egg to its own diffusion gradients

8 When the medium is made more basic, the percentage of the eggs which form rhizoid protuberances toward the far end of the tube decreases to about 20 or 25 per cent between pH 8.1 and 8.6. The response of the egg to the gradients which it produces is thus statistically reversed. The determination of the polarity of the eggs by the diffusion gradients does not become as complete in alkalized as in acidified sea water

9 When the pH of the sea water is elevated to 9.1 or 9.2, salts precipitate out. The type of development is altered and the control of the diffusion gradients over the polarity of the eggs decreases

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# THE RATE OF CO<sub>2</sub> ASSIMILATION BY PURPLE BACTERIA AT VARIOUS WAVE LENGTHS OF LIGHT

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INTRODUCTION

algae has also been claimed without justification to show that the red pigment acts as a photosensitizer. These speculations apply by analogy to green plant photosynthesis where the function of the carotinoids is still an unsolved though fundamental question. Gaffron (1934) found that photosynthesis took place rapidly with infrared alone showing that light absorbed only by the green pigment can act photosynthetically as is the case in green plants (Warburg and Negelein, 1923). It is the purpose of this paper to show that only the green pigment, bacteriochlorophyll, is the light absorber for bacterial photosynthesis. In the experiments of Roelofsen, using known intensities a better yield was obtained at the wave length which was absorbed more by the green than by the red substance in his purple sulfur bacteria.

We will discuss first the absorption spectrum of the live bacteria, and notice the location of the bands due to the red and to the green pigments. Then, we will show that the action spectrum for CO<sub>2</sub> assimilation has bands that correspond in position only to those of the bacteriochlorophyll. Thus it becomes very clear that whatever the function of carotinoids, it is not photosensitization for CO<sub>2</sub> reduction.

### *Principles*

The action method of determining the absorption curve of a photosensitizing pigment in cells in thin suspensions has been described by Warburg and Negelein (1928*a*), in their work on the respiratory enzyme of yeast. Here the same principles are used to study the photosynthetic pigment in purple bacteria.

In a thin layer of homogeneous solution containing a dissolved colored substance, the amount of monochromatic light energy absorbed per unit time is given as follows

$$-\Delta I = E = I_0 \epsilon \beta q \Delta d \quad (1)$$

where  $I_0$  is the incident intensity,  $\epsilon$  the concentration of the absorbing substance,  $\beta$  its absorption coefficient,  $q$  the cross-section area, and  $\Delta d$  the thickness of the layer.

If the molecules undergo a photochemical reaction after absorbing light

$$\gamma = \frac{E}{W} \quad (2)$$

where  $E$  is the energy absorbed expressed in quanta per unit time,  $W$  is the number of molecules changed per unit time,  $\gamma$  is then the number of quanta required to change one molecule presumably a constant which is independent of wave length

In the experiments on *Chlorella* by Warburg and Negelein the quantum yield did not vary appreciably with wave length. A similar case has been found in the activation of  $O_2$  by chlorophyll under the influence of visible light by Gaffron (1927). This assumption is made in the determination of the absorption spectrum of the respiratory enzyme by Warburg and Negelein (1928a) and shown to be correct by their experiments on the dissociation of CO ferrocystein and of CO hemochromogen (Warburg and Negelein (1928b)). For the assimilation of  $CO_2$  with H by *Streptococcus varians*  $\gamma = 4$  but it has not been determined in *Spirillum rubrum*.

Combining the two expressions

$$E = \gamma W = I_0 \beta q \Delta d \quad (3)$$

$I_0$  being expressed in quanta. If  $I' =$  incident intensity in ergs, then

$$W = \frac{c \beta q I' h C \Delta d}{\gamma \lambda} \quad (4)$$

where  $h$  is Plank's constant and  $C$  the speed of light

Evaluating and converting to calories

$$W = \frac{c \beta q I_1 \Delta d}{\gamma} \times \frac{6.55 \times 10^{-27} \times 3 \times 10^{10}}{\lambda \times 10^{-7} \times 4.18 \times 10^7}$$

$$W = \frac{c \beta q I_1 \Delta d}{\gamma} \times \frac{4.71}{\lambda} \times 10^{-18}$$

where  $\lambda$  is expressed in  $m\mu$

Solving for  $\beta$

$$\beta = \frac{\gamma W \lambda}{I_1 c q \Delta d} \times 2.13 \times 10^{18}$$

In applying this equation to a thin suspension of bacteria or other cells in water we need only make the assumption that the light in

tensity is not very different in different parts of a single cell, that is, that a beam of light in passing through one bacterium is not greatly weakened. If  $\Delta d$  is so small that all parts of all the bacteria receive practically equal monochromatic intensities (*i.e.*, small absorption by the suspension as a whole), then the amount of energy absorbed will be equal to the intensity  $\times$  the exposed area  $\times$  the thickness  $\times$  the concentration of pigment in the suspension  $\times$  the absorption coefficient of the pigment. For the absolute measurement of quantum yields, all these quantities need to be evaluated, but for purposes of comparing the relative absorption coefficients for different wave lengths, it suffices to hold all terms but  $I$  and  $\lambda$  constant during one experiment. The measured relative rates at different wave lengths will then be proportional to  $\beta$  (Warburg and Negelein, 1928*b*).

The expression then reduces to

$$\beta K' = \frac{W\lambda}{I_1} \text{ or expressing } I_0 \text{ in quanta } \beta K = \frac{W}{I_0}$$

It has been found experimentally (Figs 4 and 5) that in the range of intensities used,  $W$  is a linear function of  $I_1$  wave length being constant. The intensity,  $I_1$ , falling on, and for the most part passing through, the suspension is measured bolometrically.  $W$  is determined by the manometric measurements of CO<sub>2</sub> assimilated. Using the assumption that the quantum efficiency,  $\frac{1}{\gamma}$  (whatever its value) is constant within the spectral range concerned,  $\beta K$  is thus measured for a number of wave lengths.

## EXPERIMENTAL

### *The Absorption Spectrum of the Pigments in the Living Bacteria*

Pure cultures of *Spirillum rubrum* (strain S1), kindly given us by Professor C. B. van Niel were grown in the following medium about 2 days at 35° in the light with 5 per cent CO<sub>2</sub> in argon as described elsewhere (French, 1937)

K H CO <sub>3</sub>	0.5	per cent	(0.05 M)
K butyrate	0.126	" "	(0.01 M)
NH <sub>4</sub> Cl	0.1	" "	
KH <sub>2</sub> PO <sub>4</sub>	0.05	" "	
MgCl <sub>2</sub>	0.02	" "	
Yeast autolysate	2.0	" "	

The culture was centrifuged and the bacteria suspended in tap water. Two identical absorption vessels were filled with a suspension and the absorption of both was found to be identical when measured with a photoelectric spectrophotometer. One vessel kept cool with running water was then placed close to a quartz mercury arc for about an hour to bleach the pigment in the cells. Measurements of absorption of the pigments were then made at various wave lengths.

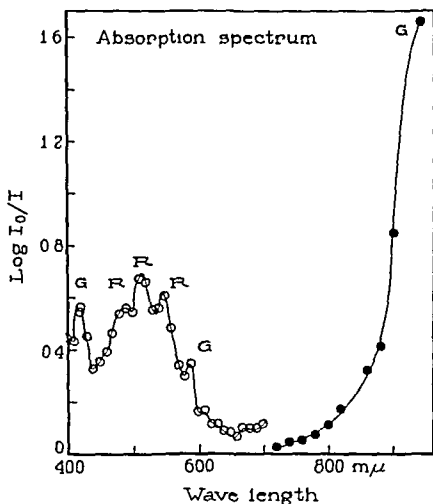


FIG. 1. The relative absorption curve of the pigments of *Spirillum rubrum* in the living bacteria measured photoelectrically using a scattering control of bleached bacteria. The maxima marked with letter R are those of the red pigment, spirilloxanthin while those labelled G are due to the green bacteriochlorophyll. Infrared wave lengths may be inaccurate due to the small dispersion of glass in this region.

by using the bleached suspension for a scattering control. The vessel was placed directly in front of the photocell to collect a large part of the scattered light. The curve of Fig. 1 presents the combined results of two such experiments. As the monochromators used for this measurement did not have sufficient dispersion for accurate work in infrared a spectrogram was made in this region to determine more precisely the band position. Fig. 2 shows one prominent band

in the near infrared with a maximum at 880  $m\mu$  and suggests a very faint one at 800  $m\mu$ . The spectrum of *Streptococcus varians* is also shown for comparison. It has bands at 860 and 800  $m\mu$  (French, 1937).

Moist bacteria extracted in the dark at 0° with methyl alcohol give up the bacteriochlorophyll, but retain nearly all the carotinoid which is, however, easily extractable with acetone. In Fig. 3, we see a relative absorption curve of such a bacteriochlorophyll extract prepared in the dark at 0° and measured with very dim light. A few

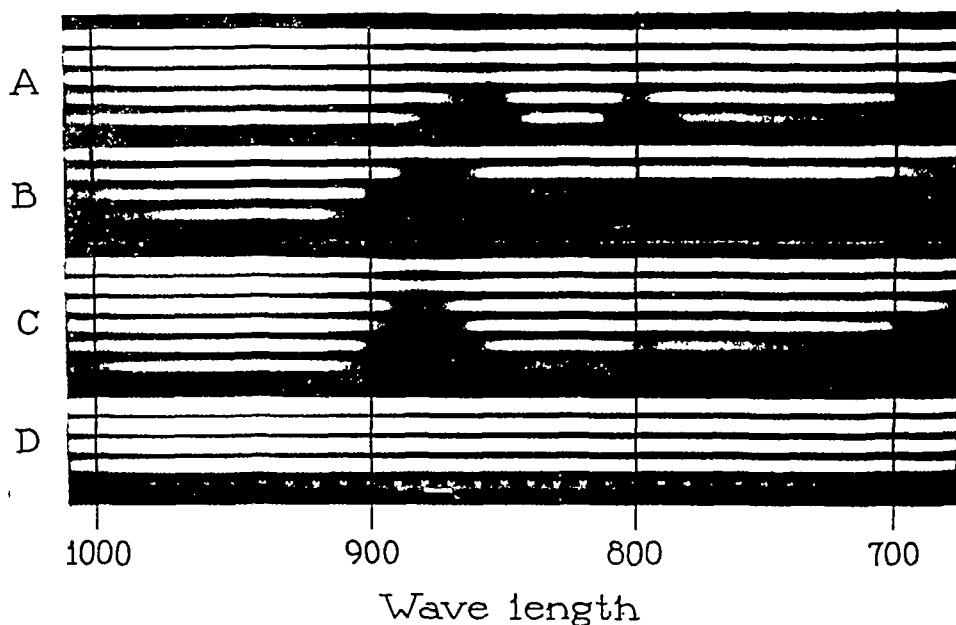


FIG. 2. Infrared spectrograms of living *Spirillum rubrum* (B and C) as compared with *Streptococcus varians* (A). (D) is the light alone without any bacteria. Taken with a Zeiss spectrograph for chemists using the grating.

minutes in the ordinary light of a room turns this solution from a clear light green to brown. The large near infrared band is shifted by extraction from 880  $m\mu$  in the bacteria to 770  $m\mu$  in methyl alcohol. The yellow band is shifted from 590 to 605. It is interesting to note that these bands are not both displaced in the same direction. From this figure, we can identify the bands marked with letter G in Fig. 1 which belong to the green pigment. They are at 880, 590, and 420  $m\mu$  in the living cells, and 770, 605, and 400  $m\mu$  in the methyl alcohol

extract The bands at 550, 515, 490  $m\mu$ , marked *R*, can easily be identified as belonging to the red pigment described by van Niel and Smith who give an absolute absorption spectrum in  $CS_2$  of the red pigment from this same strain of bacteria

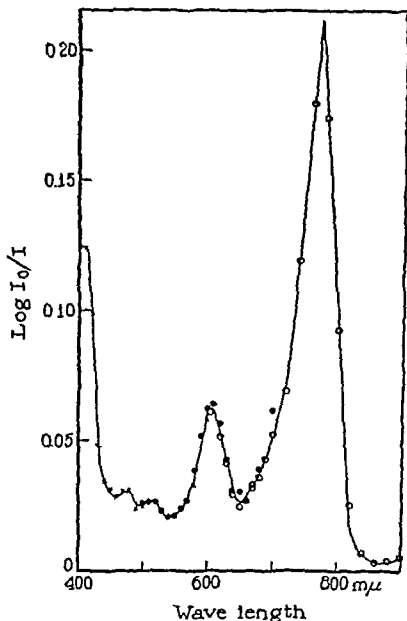


FIG 3 Relative absorption curve of the green pigment in methyl alcohol 0.2 cc moist cells extracted in the dark at 0 with 5.2 cc. absolute methyl alcohol The extract was kept in the dark and measured at room temperature with very weak light

#### *Monochromatic Light Sources*

The light used in making the photochemical action curve was obtained from a mercury lamp a sodium lamp or from a filament lamp in which case the light was dispersed by prisms and certain spectral regions isolated with a slit With



the metallic vapor lamps, the filters given by Kubowitz and Haas (1932) were used for isolating single lines. Since the experiment was performed, it has appeared that the Osram caesium tube has abundant intensity at 852 and 894  $m\mu$  (French, 1937). It may also be possible to use the Na line, 819  $m\mu$ , for similar experiments.

The instrument designated in Table II as the two prism monochromator has an opening of 1:1 with usable prism faces of 10 cm. and was built for work in the visible region by B. Halle, Nachfolger, Berlin. The light from the single coil lamp passed through a quartz condenser was thereby made nearly parallel, then converged on the slit with a crown glass lens similar to the collimator inside the instrument. All three of these lenses have an opening of about 1:1. To obtain greater dispersion in the near infrared, the instrument was built over, adding two more prisms and disposing of the entrance slit and the three large aperture lenses. A 160 mm. focus collimator larger than the prisms collected the light from a narrow coil filament placed at its focus. This arrangement was similar in principle to the apparatus used for red light by Warburg and Negelein (1923).

An image of the filament coil was spread over the plane of the curved exit slit by the original telescope lens which was retained in the instrument. The lamp, collimator lens, and first prism were mounted together on an optical bench which was supported by a pivot below the inner corner of the first prism. The other end was supported by the table and carried a pointer sliding over a fixed linear metal scale. The third and fourth prisms were those of the original instrument movable in relation to each other by a screw bearing a drum and scale. Empirically a relationship between the two scales was found which kept the telescope lens evenly illuminated. This was essential as a monochromatic image of it was cast by lenses and mirrors beyond the slit on the plane of the experimental vessel in the thermostat. Wave length calibration was carried out in the visible with a hand spectroscope checked against mercury lines. The scale readings were plotted on dispersion paper and extrapolated to include the infrared. The dispersion then was roughly 546–578, 4.8 mm., 578–692, 5.2 mm., 692–773, 4.9 mm., and the minimal region which could be isolated by an infinitely narrow exit slit at 500, 15  $m\mu$ , at 550, 20  $m\mu$ , at 600, 23  $m\mu$ , at 650, 30  $m\mu$ , at 700, 40  $m\mu$ , and at 900, 60  $m\mu$ .

The region isolated by a definite slit width was taken from the dispersion curve as twice the slit width and is shown by the bars in Fig. 6. The intensity could be diminished by various amounts using neutral glass filters.

Before or after the experiment, the light beam was concentrated into the bolometer by shifting the position of the lenses and the total energy entering the thermostat thus determined. During an experiment, constancy was checked by an ammeter in series with the lamp. The diameter of the light circle at the plane of the manometer vessel was measured by marking in pencil on ground glass held in a brass frame. After correcting for the reflection and water absorption, the total energy divided by the area over which it is spread gives the intensity incident on the bacteria. Water absorption was calculated from the

values given in Landolt Börnstein which were checked for 850 m $\mu$  with water from the thermostat. The length of light path from the quartz entrance window to the vessel was 15 cm so that about half the light of wave length 850 was absorbed before reaching the suspension.

We then have the intensity

$$I_1 = \frac{21,300}{\Omega} \times \frac{T \cdot R}{B} \times 10^{-3} \text{ cal./cm}^2/\text{min}$$

$21,300 \times 10^{-3}$  is a constant evaluated in the bolometer calibration,  $\Omega$  = ohms required to bring the galvanometer pointer back to zero when the bolometer is irradiated,  $T$  = fractional transmission of 15 cm water for the wave length measured,  $R$  = reflection, and mirror loss factor = 0.875,  $B$  = area of light beam at the position of the vessel.

We may express the light intensity in quanta per minute entering the vessel ( $I_0$ ) by a conversion factor derived as follows

$$\begin{aligned} 1 \text{ quantum} &= h\nu \text{ ergs} = \frac{hc}{\lambda} \text{ ergs} = \frac{hc}{\lambda \times 4.17 \times 10^7} \text{ cal.} \\ &= \frac{6.55 \times 10^{-27} \times 3 \times 10^{10}}{\lambda \times 4.18 \times 10^7} \text{ cal.} \end{aligned}$$

Converting  $\lambda$  from centimeters to m $\mu$  and solving

$$1 \text{ quantum} = \frac{4.70 \times 10^{-17}}{\lambda} \text{ cal.}$$

$$\text{or} \quad 1 \text{ cal} = \lambda \times 2.13 \times 10^{16} \text{ quanta}$$

Since the area of the vessel is 20.5 cm<sup>2</sup> the number of quanta entering per minute is

$$I_0 = I_1 \times 20.5 \times 2.13 \times 10^{16}$$

### *The Rate of Photosynthesis at Different Wave Lengths*

The handling of the cells was fairly similar to the procedure described elsewhere for quantum yield experiments. The same vessels were used, though the control and experimental vessels were interchanged on the manometer arms. For these experiments the bacteria after being centrifuged were suspended in the growth medium

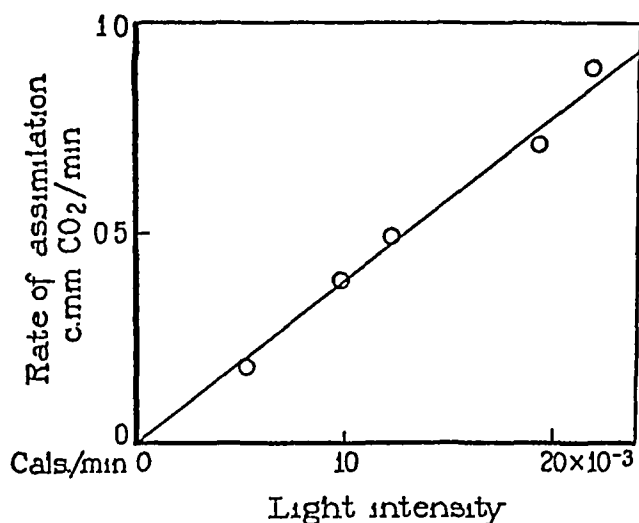


FIG 4 Rate of CO<sub>2</sub> assimilation as a function of light intensity Large silvered differential manometer, 10 cc liquid containing 259 c mm cells Gas 10 per cent CO<sub>2</sub> in argon Wave length band 825-865 mμ isolated with the four prism monochromator Area of light beam 30.5 cm<sup>2</sup>, area of vessel bottom 19.2 cm<sup>2</sup> Light intensity given in calories entering the vessel per minute Assuming total absorption, apparent  $\gamma = 17$  quanta per CO<sub>2</sub> disappearing from the gas phase

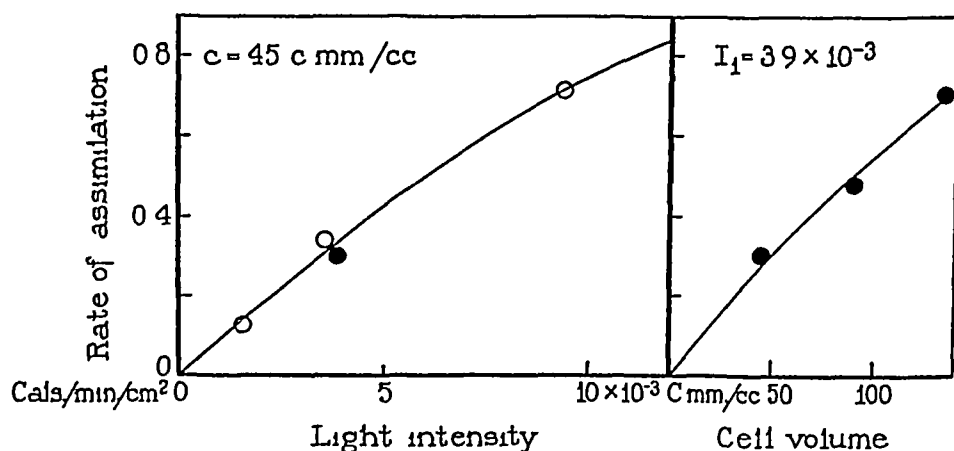


FIG 5 Rate of CO<sub>2</sub> assimilation as a function of visible light intensity and density of suspension Light from a 10 ampere filament lamp filtered through 6 cm water + 3 cm 25 per cent FeSO<sub>4</sub> in 10 per cent H<sub>2</sub>SO<sub>4</sub> Cells in 0.033 M KHCO<sub>3</sub> + 0.01 M K butyrate in tap water

at the desired concentration and assimilation of  $\text{CO}_2$  measured in an atmosphere of 5 per cent  $\text{CO}_2$  in argon. In the dark, there was a high fermentation rate. Assimilation was measured as an apparent decrease of this fermentation in the light. Dark readings were taken between the illumination periods. The vessel constant  $k_{\text{CO}_2}$  was 2.16 at the experimental temperature of  $25^\circ\text{C}$  with 6.6 cc of suspension. The number of molecules of  $\text{CO}_2$  removed per minute from the gas phase is given by

$$W = (\Delta h - \Delta h_{\text{dark}}) \times \frac{2.16}{5} \times \frac{6.06 \times 10^{23}}{22.4 \times 10^3}$$

$$= (\Delta h - \Delta h_{\text{dark}}) \times 1.17 \times 10^{16}$$

TABLE I

	Experiment 1	Experiment 2
Cell volume ( $c_v$ )	150 c.mm	150 c.mm
Liquid volume ( $V_1$ )	6.60 cc.	6.60 cc
Experimental vessel volume ( $V$ )	23.15 cc.	23.15 cc
Control vessel volume ( $V'$ )	19.37 cc	19.37 cc
Temperature ( $t^\circ$ )	$25.0^\circ\text{C}$	$25.0^\circ\text{C}$
Gas	5 $\text{CO}_2$ /argon	5 $\text{CO}_2$ /argon
Vessel constant ( $K_{\text{CO}_2}$ )	2.16	2.16
Area of light beam ( $B$ )	$38.5 \text{ cm}^2$	$36.5 \text{ cm}^2$
Area of vessel bottom	$20.5 \text{ cm}^2$	$20.5 \text{ cm}^2$
Reflection correction factor ( $R$ )	1.0	1.0

Approximate initial proportionality between intensity, density of suspension, and rate of assimilation is shown in Figs 4 and 5. The experiment summarized in Fig 5 was performed with Mr. Erwin Haas.

The rate of photosynthesis of a thin suspension was measured with a number of different wave lengths and the mols of  $\text{CO}_2$  disappearing from the gas phase per incident quantum tabulated. No account is taken of the  $\text{CO}_2$  change in the solution and the amount of absorption is not known, the data only give the relative absorption spectrum of active pigment and must not be used for efficiency calculations. The data are presented in Tables I and II, and plotted in Fig 6. Repeated attempts with Mr. Haas to measure the absolute quantum yield at  $589 \text{ m}\mu$  by extracting the pigment and measuring its absorption in methyl alcohol were not significant, even when the shift on extraction

TABLE II

<i>t</i> Time	$\lambda$ Wave length	$T_{\text{water}}$	$\omega$ Resist- ance	$I_i$ Intensity	$\Delta h$ Pressure change	$\Delta h$ dark Dark rate (inter- polated)	$\Delta h$ dark Assimila- tion	$I_0$ Incident light	$W$ Assimilation	$\beta K = W/I_0$ Light action (relative absorption spectrum)
Experiment 1 Vapor lamps with filters										
<i>min</i>	<i>mμ</i>		<i>ohms</i>	<i>cals/cm<sup>2</sup> /min × 10<sup>8</sup></i>	<i>mm/5 min Δh</i>	<i>mm/5 min</i>	<i>mm/5 min</i>	<i>quanta/min</i>	<i>molecules/min</i>	
5	578	1	403	1.37	(1 12) {1 33} 1 26 {1 20}	2 37	1 11	3 46 × 10 <sup>17</sup>	1 30 × 10 <sup>16</sup>	0 0376
10	Hg lamp									
15	Dark			0	{2 32} 2 25 {2 18}					
30										
35										
50	546	1	320	1.70	(1 20) 1 20 {1 21}	2 22	1 02	4 05 × 10 <sup>17</sup>	1 19 × 10 <sup>16</sup>	0 0269
55	Hg lamp									
70	Dark			0	{2 18} 2 19 {2 19}					
75										
110	436	1	345	1.58	(1 60) 1 61 {1 61}	2 07	0 46	3 01 × 10 <sup>17</sup>	0 54 × 10 <sup>16</sup>	0 0180
115										
130	Dark			0	{2 04} {1 88} 1 94 {1 91}					
135										
140										
155	589	1	339	1.60	{0 04} 0 07 {0 11}	1 81	1 74	4 11 × 10 <sup>17</sup>	2 04 × 10 <sup>16</sup>	0 0496
160	Na lamp									
		Filter 3 cm 25 per cent FeSO <sub>4</sub> in 10 per cent H <sub>2</sub> SO <sub>4</sub>								

[illegible]

## Two prism monochromator

300	500	1	487	1 12	{1 02}	1 51	0 46	$2.44 \times 10^{17}$	$0.54 \times 10^{16}$	0 0221
305	(470-540)				{1 09}					
320	Dark				{1 46}					
325					{1 44}					
340	675	1	397	1 37	{0 80}	1 42	0 57	$4.04 \times 10^{17}$	$0.67 \times 10^{16}$	0 0166
345	(640-710)	Filter RG2 (Schott)			{0 90}					
360	Dark				{1 39}					
365					{1 39}					

TABLE II—Concluded

$t$ Time	$\lambda$ Wave length	$T_{\text{water}}$	$\omega$ Resist- ance	$I_1$ Intensity	$\Delta h$ Pressure change	$\Delta h_{\text{dark}}$ Dark rate (inter- polated)	$\Delta h \Delta h_{\text{dark}}$ Assimila- tion	$I_0$ Incident light	$W$ Assimilation	$\beta K = W/I_0$ Light action (relative absorption spectrum)
Experiment 2 Four prism monochromator										
min	m $\mu$		ohms	cals/cm <sup>2</sup> /min $\times$ 10 <sup>8</sup>	mm/5 min $\Delta h$	mm/5 min	mm/5 min	quanta/min	molecules/min	
5	650	1	327	1.78	{2.02} {2.02}	2.39	0.37	$5.05 \times 10^{17}$	$0.43 \times 10^{16}$	0.0085
10	(625-680)				{2.03} {2.02}					
20	Dark				{2.35} {2.35}					
25					{2.37} {2.35}					
30					{2.33} {2.33}					
40	750	0.65	285	1.33	{1.46} {1.48}	2.39	0.91	$4.35 \times 10^{17}$	$1.06 \times 10^{16}$	0.0244
45	(720-780)	Filter RG2 (Schott)			{1.50} {1.48}					
55	Dark				{2.36} {2.41}					
60					{2.47} {2.41}					
85	Dark				{2.46} {2.45}					
90					{2.50} {2.45}					
95					{2.38} {2.38}					
105	900	0.35	455	0.45	{1.36} {1.28}	2.39	1.11	$1.77 \times 10^{17}$	$1.30 \times 10^{16}$	0.0735
110	(865-940)	Filter RG2 (Schott) + 5 cm water			{1.20} {1.20}					
120	Dark				{2.38} {2.33}					
135	850	0.53	555	0.56	{2.27} {2.33}	2.39	1.25	$2.08 \times 10^{17}$	$1.46 \times 10^{16}$	0.0700
140	(826-863)	Filter RG2 (Schott) + 5 cm water			{1.14} {1.14}					
					{1.15} {1.14}					

of the position of the maximum was taken into account. Apparently, large numbers of quanta (about 15) were always required to reduce one CO. Probably the high fermentation rate in the dark interfered with this measurement.

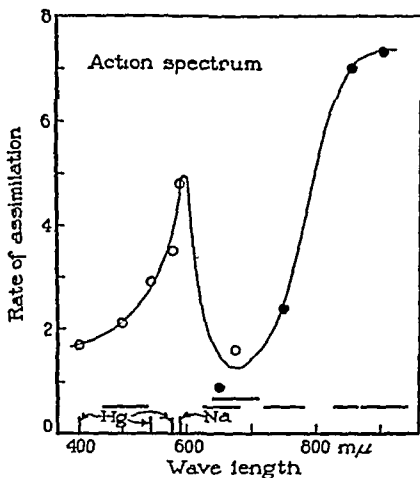


FIG. 6 Rate of  $\text{CO}_2$  assimilation of a very dilute suspension as a function of wave length of incident light. Rate scale represents molecules  $\text{CO}_2 \times 100$  per incident quantum. This curve is in effect the relative absorption curve of the photosensitizing pigment.

#### DISCUSSION

The carotinoids do not play a photosensitizing rôle in bacterial metabolism. Therefore, some other factor than photosynthetic activity must have been the cause of bacteria collecting at the red pigment, as well as at the green pigment, absorption bands in the excellent experiments performed long ago by Engelmann (1888) and confirmed by Buder (1919). There is no doubt as to the correctness of their observations, and apparently the effects observed are due to oriented swimming of the bacteria and not merely to an increased growth rate.



in certain wave lengths. Experiments on phototaxis as a function of wave length by Blaauw (1909), Castle (1931), Johnston (1934), and Voerke (1933), show that for this process, blue light is most effective. It would then be reasonable to expect that in the spectrum projection experiments purple bacteria would collect only at the bands of the carotinoids. In other work with motile photosynthetic green algae, Engelmann (1882) found that the algae moved toward a higher O<sub>2</sub> concentration along a gradient set up by O<sub>2</sub> liberation from the algae themselves when in red light. Thus, an apparent phototaxis to wave lengths of light most useful for photosynthesis turned out to be only a masked chemotaxis having nothing to do with orientation to light. It is perhaps possible that the collection of bacteria in infrared and at their 590 mμ band is nothing more than a chemotaxis in a CO<sub>2</sub> gradient caused by CO<sub>2</sub> utilization in light which is most active for assimilation.

*Spirillum rubrum* has one infrared band at about 880, and possibly one at 800, while *Streptococcus varians* has one at 860 and another smaller one at 800. Quantitative determinations of the absorption in the near infrared are needed on all available strains of photosynthetic bacteria to find out if these differences indicate different pigments in the various species.

#### SUMMARY

1 The relative absorption spectrum of the pigments in their natural state in the photosynthetic bacterium *Spirillum rubrum* is given from 400 to 900 mμ. The position of the absorption maxima in the live bacteria due to each of the pigments is: green pigment, 420, 590, 880, red pigment, 490, 510, 550.

2 The relative absorption spectrum of the green pigment in methyl alcohol has been determined from 400 to 900 mμ. Bands at 410, 605, and 770 mμ were found.

3 The wave length sensitivity curve of the photosynthetic mechanism has been determined and shows maxima at 590 and about 900 mμ.

4 It is concluded that the green bacteriochlorophyll alone and not the red pigment can act as a light absorber for photochemical CO<sub>2</sub> reduction.

This work was made possible by Professor Otto Warburg, to whom I wish to express my deepest thanks for the laboratory privileges and for his constant interest and advice. To Mr. Erwin Haas, I am very grateful, for some of this work was done jointly with him and his help many times assured the success of an experiment. It is a pleasure to thank Professors Robert Emerson and C. B. van Niel for their criticism of the manuscript.

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# EXCITATION THEORIES OF RASHEVSKY AND HILL

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(Accepted for publication, April 23, 1937)

Blair (1932) proposed the equation

$$dp/dt = KI - kp \quad (1)$$

to describe the change of the excitatory process of nerve,  $p$ , under the action of a current  $I$ .  $K$  and  $k$  are constants, and action results when  $p$  exceeds some threshold value  $h$ . The equation fits extensive experimental data but is quite unable to account for the anodic excitation at break and for non excitation by slowly rising currents. Rashevsky (1933) added a parallel equation for an inhibitory process, or threshold rise,

$$de/dt = KI - k(e - e) \quad (2)$$

$$di/dt = MI - m(i - i) \quad (3)$$

where  $K$ ,  $k$ ,  $M$ , and  $m$  are constants,  $e$  the excitatory process, and  $i$  the inhibitory one. Action results when  $e \geq i$ , and for  $m \ll k$  and  $K/k \leq M/m$  ( $M \ll K$ ), the negative process, that is, slower than the positive one, these equations satisfy the two phenomena not covered by Blair's treatment as well as those which are. These equations can also be given a physical interpretation in terms of the migration of two antagonistic ions,  $e$  and  $i$  representing their respective concentrations.

Hill (1936) proposed another set of equations, based on Blair's equation for the excitatory process but assuming that the negative one, or threshold rise, is a function of the magnitude of the excitatory process at any instant rather than of the magnitude of the stimulating current—as assumed by Rashevsky. The differential equations he implies are<sup>1</sup>

<sup>1</sup>I have substituted  $k'$  for Hill's  $k$  to avoid confusion with Rashevsky's

$$dV/dt = bI - (V - V_o)/k' \quad (4)$$

$$dU/dt = \beta(V - V_o) - (U - U_o)/\lambda \quad (5)$$

where  $V$  is the excitatory process,  $U$  the threshold, and  $b$ ,  $k'$ ,  $\beta$ , and  $\lambda$  constants, with  $\lambda \gg k'$ . Action occurs when  $V$  equals or exceeds  $U$ .

Though Hill's equations describe a physical picture of inhibition or accommodation somewhat different from Rashevsky's, it can be shown that both treatments lead to identical equations for strength-duration curves obtained with any form of stimulating current varying as an arbitrary function of time.

Integration of (4) and (5) gives respectively

$$V = V_o + be^{-t/k'} \int_{\theta=0}^{\theta=t} Ie^{\theta/k'} d\theta \quad (6)$$

$$U = U_o + \beta e^{-t/\lambda} \int_{\theta=0}^{\theta=t} (V_{\theta} - V_o)e^{\theta/\lambda} d\theta \quad (7)$$

where  $V_{\theta}$  is the instantaneous value of  $V$  at time  $\theta$ . Substitution of (6) in (7), with appropriate change in the argument and interchanging the limits of integration gives, after some rearrangement,

$$U = U_o + \beta b[k'\lambda/(k' - \lambda)] \left[ e^{-t/k'} \int_{\theta=0}^{\theta=t} Ie^{\theta/k'} d\theta - e^{-t/\lambda} \int_{\theta=0}^{\theta=t} Ie^{\theta/\lambda} d\theta \right] \quad (8)$$

Equations (6) and (8) give for the condition that at time  $t$ ,  $V = U$  and, therefore, action occurs

$$[(\lambda - k')/\beta\lambda k' + 1]e^{-t/\lambda} \int_{\theta=0}^{\theta=t} Ie^{\theta/k'} d\theta = (U_o - V_o)(\lambda - k')/b\beta\lambda k' + e^{-t/\lambda} \int_{\theta=0}^{\theta=t} Ie^{\theta/\lambda} d\theta \quad (9)$$

Solution of Rashevsky's equations (2) and (3) gives,

$$e = e_o + Ke^{-kt} \int_{\theta=0}^{\theta=t} Ie^{k\theta} d\theta \quad (10)$$

$$i = i_o + Me^{-mt} \int_{\theta=0}^{\theta=t} Ie^{m\theta} d\theta \quad (11)$$

and again for the condition that at time  $t$ ,  $c = i$  and action occurs,

$$(\lambda/M)e^{-kt} \int_{\theta=0}^{\theta=t} Ie^{k\theta} d\theta = (i_0 - c_0)/M + e^{-mt} \int_{\theta=0}^{\theta=t} Ie^{m\theta} d\theta \quad (12)$$

Equation (12) for the strength—duration relationship derived from Rashevsky's theory is identical with the parallel equation (9) derived from Hill's, provided

$$\begin{aligned} \lambda/M &= (\lambda - k\gamma)/\beta\lambda k + 1 \quad (i - c)/M = (U - V)(\lambda - k\gamma)/b\beta\lambda k \\ k &= 1/k \quad m = 1/\lambda \end{aligned} \quad (13)$$

Assuming with Hill a "normal accommodation," is equivalent to putting  $\beta = 1/\lambda$  (Hill) or  $K/M = k/m$  (Rashevsky) Relationships (13) then become

$$(i - c)/M = (U - V)(\lambda - k\gamma)/bk \quad k = 1/k \quad m = 1/\lambda \quad (14)$$

Thus any prediction as to excitation by any arbitrary current form deduced on the basis of one theory can, by suitable choice of constants, be exactly duplicated by the other, and it becomes impossible to distinguish between the theories by any such experiments

I wish to express my appreciation to Dr R W Gerard who suggested this investigation

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# VISUAL ADAPTATION AND CHEMISTRY OF THE RODS\*

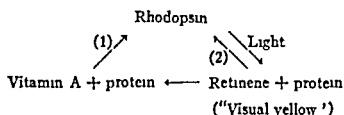
By GEORGE WALD AND ANNA BETTY CLARK

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(Accepted for publication, July 1, 1937)

Sixty years ago Kühne<sup>1</sup> concluded from direct observation of frog and rabbit retinas that visual purple may be synthesized by two processes a slow "neogenesis" from colorless initial stages, following intense and protracted irradiation, and a three to four times faster "anagenesis" from yellow initial stages, following "incomplete bleaching"

Neogenesis and anagenesis may now be identified with the formation of rhodopsin from vitamin A and from retinene respectively, as described in part in the equations (Wald, 1935-36 *a*, *b*)



The synthesis and removal of rhodopsin in the retina are reflected in the sensory phenomena of dark and light adaptation (Hecht, 1919-20) It should be possible, therefore, to test the reality of the chemical formulation *in vivo* with properly designed adaptation experiments

The chemical equations permit the following specific predictions concerning dark adaptation

1 Exposure of the dark adapted eye to a short, intense flash of light should convert a large quantity of rhodopsin to retinene, but little retinene should have had time to form vitamin A Directly following

\* A report of these experiments was presented at the Spring meeting of the American Physiological Society, 1936 A partial abstract has been published (Wald and Clark, 1936)

<sup>1</sup> Ewald and Kühne 1878, p 276, Ayres and Kühne, 1878, p 234, summarized by Kühne, 1879, p 317



such exposure, dark adaptation should depend principally upon reaction (2), and so should be relatively rapid

2 Long illumination of the eye should bring the visual cycle to a steady state. Dark adaptation following such exposure should contain a maximal contribution from reaction (1), and so should be relatively slow

3 Since retinene is removed to form vitamin A in addition to rhodopsin, dark adaptation as it proceeds should depend increasingly, and finally entirely, upon reaction (1). The latter portions of dark adaptation, therefore, should display the comparatively simple character of the slow process alone

Our experiments verify these predictions. Between given initial and final thresholds the rods may dark adapt along many paths, depending upon the period and intensity of the preceding light adaptation. The early portions of dark adaptation are rapid following short, slow following long irradiation. As dark adaptation proceeds, the slow process grows increasingly prominent, and occupies completely the later stages of adaptation.

The complete description of light adaptation includes these phenomena. Following increasing lengths of exposure to light of constant intensity, the visual threshold rises, as the concentration of rhodopsin decreases. Independently, the speed of dark adaptation falls, as the proportion of vitamin A to retinene rises. Both variations cease as the visual cycle attains a steady state, the end of the light adaptation process.

## I

### *Methods*

*Light Adaptation*—The subject faced an opal glass screen  $18.3 \times 23.5$  cm placed about a foot from the eyes. This transmitted the illumination from a 1000 watt lamp mounted upon a track. Brightness was varied by moving the lamp toward and away from the screen. No artificial pupil was used.

In most experiments the two eyes were adapted to different brightnesses and for different times. For this the subject wore goggles, over either window of which a blind or a neutral filter could be fitted. To adapt the eyes for different periods, the blind was placed over one eye during the initial portion of a light adaptation, and removed for its final portion. To adapt the eyes to different brightnesses, a neutral filter was fitted over one eye, the other receiving the full

illumination from the screen. Both procedures were frequently combined. One eye, fitted with a filter, was adapted for a long period to a low intensity, the other eye, darkened for the first portion of the irradiation, was exposed for a shorter interval to the full adapting intensity.

*Dark Adaptation*—Periodically during dark adaptation, the threshold of either eye was measured, using a circular test field  $0.44^\circ$  in diameter, located  $9.8^\circ$  to the right of a small, bright fixation point. The latter was illuminated continuously, the field itself exposed for short flashes by means of a camera shutter operated by the subject and set at 1/50 second. The intensity of the test field was regulated with the adaptometer of Derby, Chandler, and Sloan (1929), loaned to us through the courtesy of Dr. J. Herbert Waite of Boston. In this instrument the illumination is varied by means of a pair of neutral circular wedges rotating in opposite directions so as to compensate each other. The adaptometer was mounted in a special assembly and was recalibrated by Dr. C. P. Winsor of this Laboratory.

*Procedure*—The subject was left in complete darkness for 15 to 30 minutes depending upon previous exposure to light. She was then light adapted for the prescribed interval. At its close the adapting light was turned off. The observer, having fixated her eye, proceeded to flash the test illumination initially set at a sub-threshold intensity. Between clicks of the shutter the operator raised the intensity by regular steps of about 0.02 log unit, until the subject reported that the field had been seen. The operator noted time and wedge reading. Periodically this procedure was repeated. All observations were monocular, usually with alternate eyes. It was found that readings could be made as often as desired—occasionally up to twice within a minute upon a single eye—without materially disturbing the course of adaptation.

### Measurements

*Light Adaptation to Various Intensities, Long Exposures*—The dark adaptation of the subject's left eye was measured following 7 minute exposures to 30.3, 105, and 643 millilamberts. Individual threshold determinations are presented in Table I and Fig. 1.

Dark adaptation following exposure to 643 millilamberts shows a distinct break at about 4 minutes. This occurs earlier and less abruptly in the 105 millilambert curve, and is absent from the 30.3 millilambert function. Similar breaks appear in the uppermost curves of Figs. 2 and 3. They are due to transitions from cone to rod thresholds (Hecht, Haig, and Wald, 1935-36). The cone functions precede the breaks, and when present seriously hinder the experiments by preventing the measurement of initial rod thresholds. The extended smooth functions which follow the breaks are due to rods, and are the concern of the present investigation.

If rod dark adaptation curves depended upon a single process, they should all form segments of a single function. However, the curves shown in Fig. 1 vary in form, and cannot be superimposed by any displacement on the time axis. The general implications of this variation have been discussed by Winsor and Clark (1936). The first few minutes of rod dark adaptation become markedly slower as the intensity

TABLE I

Dark adaptation following 7 minutes exposure to 30.3, 105, and 643 millilamberts, and following 10 seconds exposure to 1890 millilamberts. Subject A-B-C. Time in minutes, thresholds in millilamberts.

Left eye 7 min exposure						Right and left eyes 10 sec exposure	
30.3 millilamberts		105 millilamberts		643 millilamberts		1890 millilamberts	
Time in dark	Log threshold	Time in dark	Log threshold	Time in dark	Log threshold	Time in dark	Log threshold
0.5	2.26	0.7	2.68	0.5	2.99	0.6	1.07
1.7	3.86	1.4	2.49	1.5	2.85	1.0	2.80
2.7	3.77	2.3	2.42	3.3	2.82	1.4	2.68
3.5	3.68	3.1	2.32	4.2	2.66	2.0	2.44
4.6	3.58	3.9	2.21	5.6	2.49	3.0	2.26
5.7	3.46	5.2	3.97	6.9	2.32	3.8	2.06
7.4	3.41	6.8	3.85	8.6	2.04	4.9	3.83
8.5	3.36	8.5	3.74	10.7	3.78	6.3	3.67
10.1	3.31	10.5	3.62	13.7	3.51	7.9	3.44
11.6	3.26	12.8	3.39	17.1	3.34	9.6	3.29
13.6	3.19	17.1	3.24	21.7	3.34	11.2	3.16
19.2	3.09	20.6	3.24	23.0	3.34	13.0	3.08
23.8	3.06	25.5	3.22	26.8	3.31	16.5	3.06
28.0	4.99	29.1	3.19	30.6	3.24	20.5	3.04
33.7	3.04	35.0	3.17	34.8	3.21	25.7	3.04
		36.3	3.14			30.5	3.03
						35.4	3.04

of the adapting light rises, indicating more complete dependence upon the slow synthesis of rhodopsin from vitamin A. This result is due to some precise arrangement of reaction orders in the visual cycle, and could not have been predicted from chemical information now available. Following long exposures to light more intense than about 200 millilamberts the early portions of rod dark adaptation are hidden

behind the cone function, and these changes in velocity can no longer be detected<sup>2</sup>

*Duality of Rod Dark Adaptation*—Simultaneous with the three 7 minute exposures of the subject's left eye described above, the right eye was exposed to 1890 millilamberts for 10 seconds. In an added experiment, to permit comparison of all functions in the left eye, both eyes were simultaneously adapted to 1890 millilamberts for 10 seconds. The dark adaptation curves obtained in these five experiments were

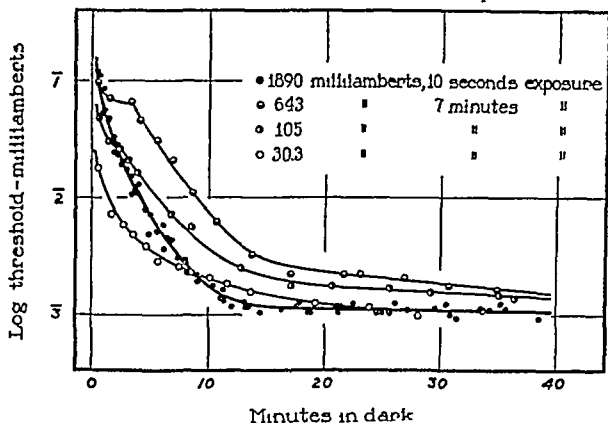


FIG 1 Dark adaptation following long exposures to moderate intensities and short exposure to a high intensity. Following 7 minute exposures rod dark adaptation is slower the higher the light adapting intensity. Following the 10 second exposure, dark adaptation is extremely rapid.

identical. The individual measurements are plotted as solid circles in Fig 1, their averages are presented in the last two columns of Table I.

Dark adaptation following 10 seconds' irradiation is very much more rapid than following long exposures. Comparison of the 10 second function with each of the 7 minute curves in Fig 1 illustrates three aspects of this relation.

<sup>2</sup>Hecht, Haig, and Chase (1936-37) have measured dark adaptation following 2 minute exposures to a wide range of intensities. The portions of their results pertinent to the present discussion agree essentially with those of Winsor and Clark and with our own experiments.

The 10 second and the 7 minute 643 millilambert curves possess the same initial thresholds, but the former reaches a constant minimum within about 15 minutes, while the latter is still incomplete after 35 minutes. This comparison is complicated by the fact that the initial thresholds of the 10 second curve appear due to rods, those of the 7 minute curve to cones, the degree of rod light adaptation was apparently very much higher in the 7 minute exposure.

The 7 minute 105 millilambert curve appears to correct this discrepancy. Its initial rod thresholds seem to coincide approximately with those of the 10 second function. Still the 7 minute curve is clearly very much slower, and adaptation remains incomplete after 35 minutes.

Finally, comparison of the 10 second with the 7 minute 30.3 millilambert function shows this distinction unequivocally. The former adaptation starts with thresholds more than five times higher than the latter, yet is so much faster that it overtakes and crosses the 7 minute curve after about 8 minutes of dark adaptation, and is complete about 8 minutes sooner. This experiment has been repeated a number of times, interchanging the light adaptations of the two eyes, with identical results.

It is clear that the length of exposure to light introduces changes in the character of rod dark adaptation for which adjustment of the intensity fails to compensate. Dark adaptation following short exposures is much more rapid than that following long irradiation. This is the result predicted from the chemical formulation.<sup>3</sup>

*Light Adaptation to Various Intensities, Short Exposures*—Dark adaptation was measured following 5 second exposures to 3.6, 12, 120, 245, 750, and 890 millilamberts and after the 0.02–0.04 second exposure to a photoflash lamp (Mazda, No. 20, 45,000 lumen-seconds). Typical results are presented in Table II and Fig. 2.

As the intensity of the adapting light falls, the speed of dark adaptation increases, progressing toward more complete dependence upon the rapid synthesis of visual purple from retinene. We were unable by lowering the intensity to drive the system into a limiting state, in which further dimming of the adapting light produced no further changes in the speed of dark adaptation. It appears impossible,

<sup>3</sup> Riggs (1937), using an electrometric procedure, has found a similar phenomenon in the "cone" dark adaptation of the frog eye.

therefore, to isolate the rapid reaction by these means This confirms a prediction from the chemical formulation Since retinene

TABLE II

Dark adaptation following 5 sec exposures to 3.6-1890 millilamberts, and following 1/50-1/25 sec. exposure to a photoflash lamp (Mazda, No 20) Subject A B C Time in minutes, thresholds in millilamberts Thresholds of the left eye are printed in bold face type

Intensity of exposure									
3.6 millilamberts		245 millilamberts		750 millilamberts		1890 millilamberts		Photoflash	
Time in dark	Log threshold	Time in dark	Log threshold	Time in dark	Log threshold	Time in dark	Log threshold	Time in dark	Log threshold
0 1	3 68	0 2	2 73	0 3	2 92	0 4	1 07	1 2	1 02
0 4	3 21	0 5	2 23	0 6	2 61	0 7	1 02	2 1	2 96
0 7	3 11	1 0	3 94	0 9	2 40	1 0	2 95	3 2	2 92
1 1	4 99	1 4	3 71	1 3	2 23	1 4	2 89	4 2	2 86
1 4	4 99	2 0	3 62	1 8	3 99	2 1	2 75	5 6	2 85
2 2	4 94	2 5	3 39	2 7	3 62	2 7	2 53	6 8	2 59
4 0	4 92	3 2	3 42	3 3	3 59	3 5	2 40	7 4	2 46
4 9	4 97	3 9	3 12	4 0	3 54	4 3	2 03	8 7	2 19
6 0	4 92	4 4	3 07	4 7	3 37	5 0	2 15	9 6	3 99
6 9	4 94	5 1	3 05	5 2	3 34	6 5	3 74	11 0	3 78
8 4	4 94	5 7	3 02	6 0	3 22	7 8	3 47	12 9	3 49
9 6	4 94	6 5	3 00	6 7	3 12	8 5	3 42	15 6	3 34
10 3	4 92	8 0	4 97	7 3	3 02	9 6	3 24	17 3	3 24
		9 3	3 00	8 3	3 07	10 4	3 14	20 5	3 19
		10 4	4 95	8 9	3 00	11 6	3 07	24 0	3 12
		12 6	4 87	10 1	3 00	12 3	3 05	29 0	3 02
		14 3	4 95	10 5	4 92	13 8	4 97	34 2	4 97
		19 7	4 92	11 8	4 90	14 9	4 95	35 7	3 00
		20 9	4 87	12 8	4 85	17 0	4 97		
		27 6	4 95	15 3	4 92	19 1	4 95		
		30 2	4 95	19 5	4 92	27 1	4 92		
		32 2	4 92	20 1	4 87	27 5	4 90		
		32 5	4 87	22 7	4 92	33 1	4 95		
		40 6	4 90	27 3	4 95	35 7	4 90		
				28 6	4 97	36 3	4 92		
				31 1	4 85	36 7	4 92		
				31 5	4 87				
				37 0	4 85				

forms vitamin A in addition to rhodopsin, every dark adaptation as it progresses depends increasingly, and finally entirely upon the slow synthesis of rhodopsin from vitamin A

This is also the explanation of the fact that at the highest intensities investigated the dark adaptation curves following short exposures do enter a limiting condition, indistinguishable from that reached at high intensities with long exposures. In the uppermost curve of Fig 2, approximately the first 6 minutes of rod dark adaptation are concealed behind the cone function. By the time the rod segment has emerged apparently all but a negligible portion of retinene has already been removed. The entire measurable portion of rod adaptation therefore possesses the simple character of the slow process alone.

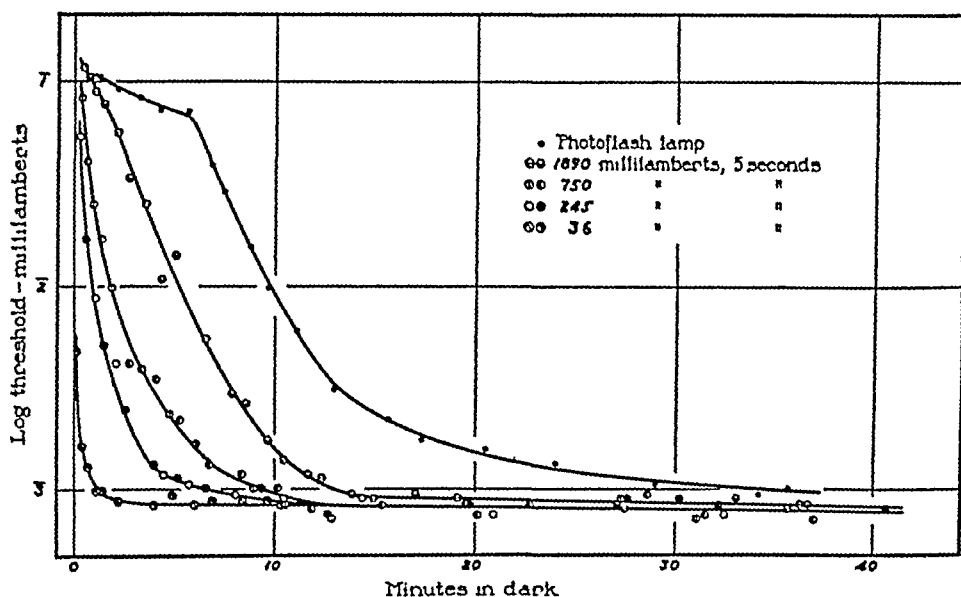


FIG 2 Dark adaptation following short exposures to various intensities. The initial portions of rod dark adaptation increase in velocity as the light adapting intensity falls.

*Various Durations of Light Adaptation, Constant Intensity*—Both eyes were exposed to 333 ml, the right eye repeatedly for 5 minutes to serve as control, the left eye for various durations from 10 seconds to 20 minutes. The data for the left eye are presented in Table III and Fig 3.

Increase in the period of light adaptation results in two changes.

1. The initial dark adaptation thresholds rise. This effect, the classic index of light adaptation, is complete within 10 minutes.

irradiation Doubling this interval produces no further change beyond the normal scatter of the function

2 The velocity of rod dark adaptation decreases In the present measurements this change appears complete after 2 minutes of light adaptation Beyond this interval, however, the initial segments of rod dark adaptation are hidden behind the cone function, and probably continue to change in form as light adaptation progresses

TABLE III

Dark adaptation following exposure to 333 millilamberts for various periods  
Subject A B C Left eye Time in minutes, thresholds in millilamberts

Exposure periods											
10 sec.		1 min		2 min		5 min.		10 min		20 min.	
Time in dark	Log thresh old	Time in dark	Log thresh old	Time in dark	Log thresh old	Time in dark	Log thresh old	Time in dark	Log thresh old	Time in dark	Log thresh old
0 4	2 06	0 35	2 77	0 3	2 77	0 6	2 75	0 35	2 92	0 4	1 05
1 3	3 87	1 1	2 26	1 1	2 46	1 3	2 65	1 0	2 88	0 9	2 97
2 5	3 44	2 0	3 91	2 1	2 21	2 4	2 57	2 0	2 86	1 3	2 97
3 5	3 14	2 5	3 86	3 1	2 06	3 4	2 46	2 9	2 80	2 2	2 92
4 8	3 14	3 4	3 72	4 3	3 87	4 5	2 38	4 2	2 72	2 8	2 86
6 0	3 10	4 5	3 54	5 4	3 76	5 7	2 19	5 6	2 49	3 7	2 83
7 6	3 10	5 7	3 47	7 0	3 52	6 8	3 97	7 8	3 99	5 0	2 62
9 2	3 07	8 0	3 17	9 6	3 32	8 6	3 66	9 4	3 69	5 8	2 41
10 1	3 05	12 6	3 05	11 3	3 12	11 9	3 34	11 8	3 54	7 1	2 14
13 0	3 00	14 3	3 05	13 5	3 12	16 6	3 22	13 4	3 42	8 7	3 91
15 7	3 02	20 1	3 07	17 7	3 05	19 5	3 19	15 2	3 32	11 6	3 57
18 3	3 00	28 2	3 00	22 0	3 07	23 4	3 14	19 6	3 22	15 0	3 37
23 9	3 00	30 4	4 92	24 6	3 12	29 2	3 14	26 2	3 10	18 2	3 22
38 1	3 00	33 5	3 02	29 2	3 00	30 5	3 07	31 4	3 10	22 9	3 15
		37 0	3 05	32 8	3 02	34 5	3 07	37 0	3 10	30 2	3 10
				35 2	3 00			40 1	3 05	35 2	3 07
				37 2	3 00						

The relation between dark adaptation and exposure to a constant intensity has been measured previously by Lohmann (1907) Rabinowitsch (1908), and Müller (1931)

Lohmann's measurements were inaccurate His dark adaptation curves show some evidence of slowing as light adaptation proceeds and appear to become constant following 6 to 10 minutes of irradiation



Rabinowitsch measured roughly no more than the first 15 minutes of dark adaptation following  $\frac{1}{4}$  minute to 2 hour exposures to a constant adapting light. She concluded that the velocity of dark adaptation decreases markedly as light adaptation progresses.

Muller has recently measured accurately the first 27 minutes of dark adaptation following 1 to 40 minute exposures to 3000 lux (about 300 millilamberts). Despite important differences in technique, his conclusions are almost identical with those stated here. The rod portion of dark adaptation was found to become practically constant in position following exposures longer than 10 minutes. Following 1, 2, and 5 minute exposures, the speed of rod adaptation decreases progressively.

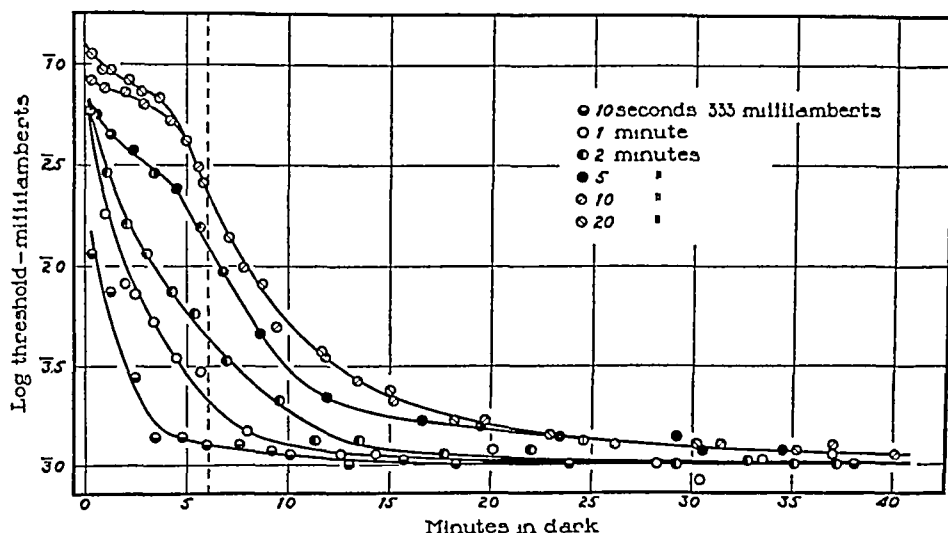


FIG 3 Dark adaptation following various lengths of exposure to 333 millilamberts. As light adaptation proceeds the visual threshold rises and independently the speed of rod dark adaptation decreases. Intercepts with these curves of the ordinate drawn at 6 minutes furnish most of the points in Fig 4.

Beyond 5 minutes exposure changes in velocity can no longer be detected, as in the present experiments this situation is associated with the concealment of the first few minutes of rod adaptation by the cone function.

*Light Adaptation*—Light adaptation is commonly defined as the rise in visual threshold which accompanies irradiation of the eye. Ideally this process should be measured by determining thresholds during instantaneous interruptions of the adapting light. Such an experiment has not yet been successfully performed. Lohmann (1907) substituted for this procedure the measurement of thresholds after

an arbitrary interval in darkness, interpolated from a series of dark adaptation curves such as Fig 3. Lohmann chose for this purpose a 10 second interval. It is clear from Fig 3 that thresholds after 10 seconds in darkness are due in the early stages of light adaptation to rods, in later stages to cones, and therefore yield no clear information concerning either type of receptor.

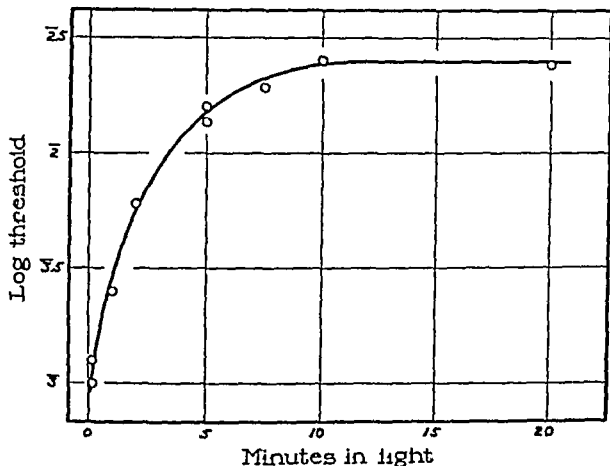


FIG 4 Light adaptation. Thresholds at 6 minutes in darkness following various lengths of exposure to 333 millilamberts.

An ordinate drawn at 6 minutes through the curves of Fig 3 intercepts only rod functions. The series of interpolated thresholds so obtained partly expresses the light adaptation of the observer's rods to 333 millilamberts. These thresholds, with a number of others similarly obtained from data not shown in Fig 3, are plotted against the period of light adaptation in Fig 4. The precise shape of the curve is of no importance, since it varies with the position of the time intercept in Fig 3, here quite arbitrarily chosen. The curve merely

expresses conveniently the fact, apparent in Fig 3, that on exposure to 333 millilamberts the rod threshold rises, at first rapidly, then more slowly, finally becoming constant in about 10 minutes

In addition to, and independently of, this change, the speed of rod dark adaptation falls as the exposure to light lengthens. This presents an added and hitherto neglected aspect of light adaptation. We may assume with Hecht that the rise in visual threshold which classically defines light adaptation corresponds with a fall in rhodopsin concentration in the rods. Then, following our chemical formulation, the decrease in speed of dark adaptation is due to increase in the proportion of vitamin A to retinene during light adaptation. The complete process of light adaptation includes both types of variation, and is expressed adequately only with a family of dark adaptation curves as in Fig 3. The steady state in which light adaptation ends is characterized by constancy both of form and position of the dark adaptation function.

It is commonly stated that light adaptation is very much more rapid than dark adaptation. This is only qualifiedly true. We have found light adaptation to 333 millilamberts to occupy about 10 minutes. Dark adaptation requires no longer, following 5 seconds exposure to light as intense as 750 millilamberts.

#### SUMMARY

1 The reality of a chemical cycle proposed to describe the rhodopsin system is tested with dark adaptation measurements.

2 The first few minutes of rod dark adaptation are rapid following short, slower following long irradiation. As dark adaptation proceeds, the slow process grows more prominent, and occupies completely the final stages of adaptation.

3 Light adaptation displays similar duality. As the exposure to light of constant intensity lengthens, the visual threshold rises, and independently the speed of dark adaptation decreases.

4 These results conform with predictions from the chemical equations.

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# STUDIES ON THE DIFFUSION EFFECT UPON IONIC DISTRIBUTION

## II EXPERIMENTS ON IONIC ACCUMULATION

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The present report furnishes experimental confirmation of the first paper of this series (1), which gave a theory for the ionic distribution in the steady state of a system of a type that may well occur in biology (*cf* Fig 1)

### *Review of the Theory*

The system considered and the theoretical results are recapitulated as follows

Across a permeable boundary  $m$  there was supposed to be present a constant difference in concentration of either the cation  $D^+$  or the anion  $A^-$ . The maintenance of this condition accomplished, for example by a continuous addition of the substance  $DA$  to the rather small volume ( $i$ ), causes  $DA$  to act as a 'diffusion agent' which steadily diffuses across  $m$  into the part ( $o$ ). In ( $o$ ) a fixed, constant composition was maintained by keeping the volume large.

It was predicted that the continuous steady diffusion of  $DA$  was bound to influence the distribution of other electrolytes present denoted by  $M^+B^-$   $M'^+B'^-$  etc., which were not participating in any active diffusion as was  $DA$ . These ions were called 'passive ions'. If, for example, the initial composition was the one graphically shown in Fig 2 *a* the ultimate effect of the steady diffusion of  $DA$  would consist in an accumulation of  $M^+$  ions and an impoverishment of  $B^-$  ions on the inside, as pictured in Fig 2 *b*. It was supposed that the  $D^+$  ions had a higher mobility than the  $A^-$  ions.

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\* The experiments were carried out when the author held a Rockefeller Foundation Fellowship

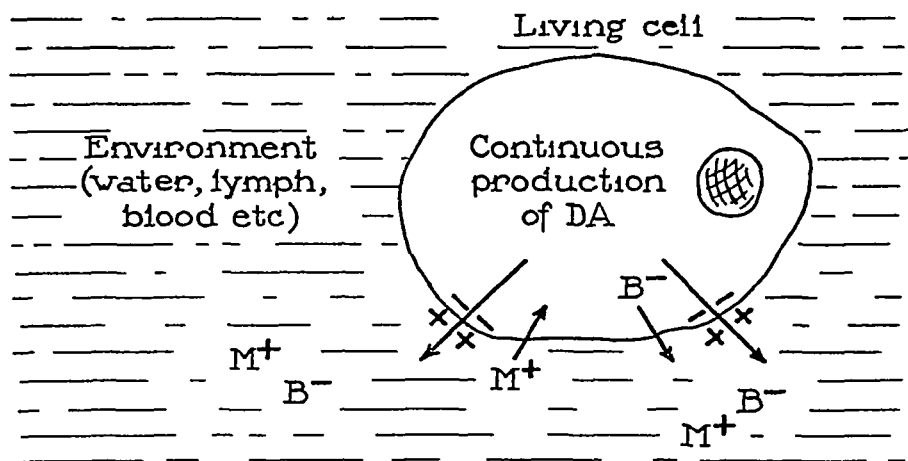
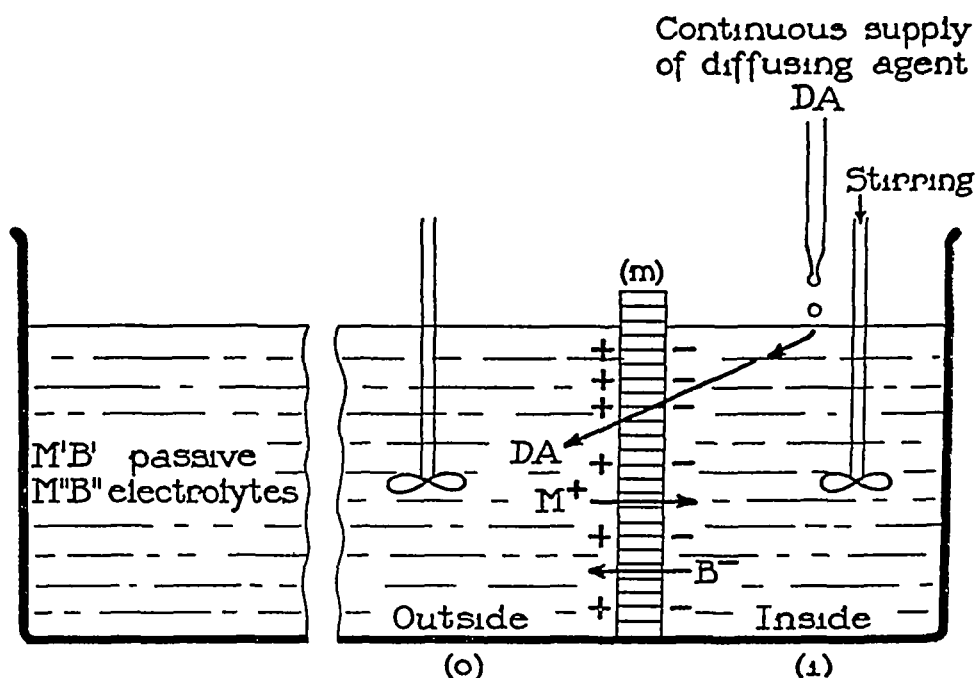


FIG 1 Scheme of the system considered The upper part shows the principle of the experimental arrangement The lower part demonstrates schematically a biological analogy to this system

The theory leads to the following alternative expressions for the distribution of the passive ions

(a) In terms of the final electrical (diffusion) potential  $\pi$  for univalent ions,

$$\log \frac{M_i^+}{M_o^+} = \log \frac{B_o^-}{B_i^-} = \frac{F}{RT} \pi \quad (1)$$

where  $M_i^+$ ,  $B_i^-$ , and  $M^+$ ,  $B^-$  denote concentration inside and outside respectively,  $F$  is the Faraday,  $R$  is the gas constant and  $T$  the absolute temperature. When  $\pi$  is expressed in millivolts,  $F/RT$  is equal to  $1/58$  (at  $18^\circ\text{C}$ )

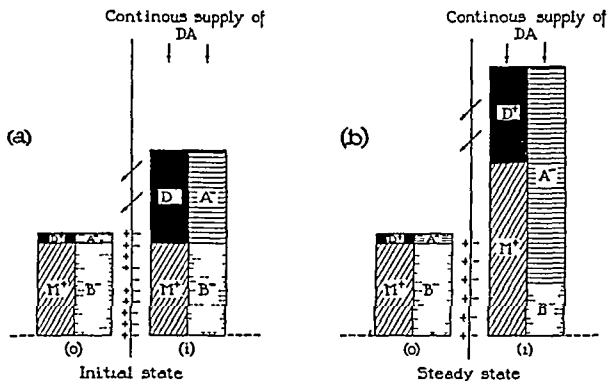


FIG. 2 The effect of a steady diffusion of  $DA$  upon the ionic distribution when  $u_D > v_A$ .  $DA$  is by some means (by addition or production) steadily supplied inside (i). The concentrations of the electrolytes outside (o) are kept constant. An electrical potential arises across the boundary (membrane). This causes an inward migration of  $M^+$  and an outward migration of  $B^-$ . Finally the concentration gradients have become sufficiently large to balance the electrical gradient and the system approaches a steady state. Thus the  $M$  ions became accumulated and the  $B$  ions diminished in amount inside.

The scale used in this figure corresponds very closely with the conditions of Experiment 1 (see Table I).

(b) In terms of known concentrations the distribution can be explicitly calculated from the following transcendental equation

$$\log \xi = \log \frac{M_i^+}{M^+} = \log \frac{B^-}{B_i^-} = \frac{u_D - v_A}{u_D + v_A} \log \frac{D^+ + \sum M_i^+}{D^+ + \sum M^+} \quad (2)$$



Here  $u_D$  and  $v_A$  denote the mobility of the  $D^+$  and  $A^-$  ions in the diffusion layer. It should be noticed that  $(D^+ + \Sigma M^+)$  is equal to the *total* concentration of the electrolytes.

Furthermore, the fact was brought forward, as is evident from Equations 1 and 2 here, that the relationship

$$\frac{M_i'^+}{M_o'^+} = \frac{M_i''^+}{M_o''^+} = \frac{B_o'^-}{B_i'^-} = \frac{B_o''^-}{B_i''^-} = \xi \quad (3)$$

must be valid. Equation 3 is immediately recognized as being characteristic also for the Gibbs-Donnan equilibrium, which obviously constitutes a special case of the more general system described by Equation 2, obtained when  $u_D$  or  $v_A$  approaches zero.

It should be emphasized that neither Equation 1 nor Equation 3 alone permits any explicit calculation of the ionic distribution. The factors which determine the distribution picture are (a) The mobility relation  $u_D/v_A$ , (b) the concentration ratio  $D_i^+/\Sigma M_o^+$ , and, (c) the concentration ratio  $D_i^+/D_o^+$ .

The further from unity  $u_D/v_A$  is and the higher  $D_i^+/\Sigma M_o^+$  or  $D_i^+/D_o^+$  is, the higher accumulation (or impoverishment) will be attained by the passive ions. This statement follows from Equation 2. In a less abstract way, it can also be expressed with the aid of Equation 1 by saying that the greater diffusion potential present across the boundary, the more marked accumulation effects (or the reverse) are obtained.

Finally it should be emphasized that this diffusion effect upon ionic distribution is *not* any kind of cataphoresis effect, because no current is flowing. Although interpretations in terms of a (diffusion) potential are rather convenient, it might be more correct to employ a concept of ionic exchange.

### Earlier Experiments

The phenomenon of accumulation and impoverishment of certain ions in a system where a steady diffusion takes place is related to a great many experimental observations.

These are described in the literature as "*Diffusion gegen das Konzentrationsgefälle*" by Behn (2) in 1897, "*Diffusion retrograde*" by Thovet (3) in 1902, "*Counterdiffusion*" by Osborne and Jackson (4) in 1914, and Walpole (5) in 1915, etc. These workers simply placed two solutions on top of one another, one containing the diffusion agent, both having another electrolyte in common. After an arbitrary time two or more parts of the diffusion column were analyzed. In general a redistribution of all ions could be observed. Apparently there was no possibility of obtaining any steady state with these conditions. The effects shown ought correctly to be classified as "temporary diffusion effects" (see (1) p. 155) and were in most cases rather small. In the best cases the differences of passive ion concentration amounted to from 15 to 20 per cent. Some experiments with condi-

tions corresponding to the theory outlined above and of the same type as will be reported here, were carried out some years ago by Straub (6). He observed an accumulation of cations and a simultaneous impoverishment of anions in a clay cylinder inside of which acid was supplied. Straub failed, however, to offer any satisfactory discussion of what he called the '*Harmome Einstellung*' of the ions, nor did his data seem to be suitable for any treatment in regard to the theory now accessible.

This subject is also related to the numerous cases of 'accelerated or retarded diffusion' of electrolytes reported, for instance by McBain and Dawson (7) (cf. also reference (8)). This is quite natural, because the theoretical considerations here advanced are founded on the basis of Nernst's (9) classical kinetic theory for electrolyte diffusion.

Summarizing the previously published works, it may be said, that there were no experiments available for use as a test for the theory proposed for a steady state.

#### EXPERIMENTAL PROCEDURE

In principle the set up was that sketched in Fig. 1: a small 'inside volume' 10–30 cc., was separated from a large 'outside' volume (0.5–1.5 liters) by a porous convection proof membrane, *m*. Both solutions were kept homogeneous up to the membrane by stirring. In (i) the diffusing agent was continuously supplied either in the form of a slow stream of a concentrated solution ( $\text{HClO}_4$ ), or by addition of the agent in a solid form so as to maintain a saturated solution ( $\text{KIO}_3$ ).

*Apparatus*—The apparatus used has been described in another paper (10). The supply of the concentrated diffusing agent in Experiment 1,  $\text{HClO}_4$ , was regulated by varying the level of a Mariotte's vessel, which through a flexible tube ending in a long capillary jet, was in communication with the inside solution. Only one membrane was employed, consisting of four superimposed layers of cellophane 600 (Du Pont Company), in wet condition of a total thickness of 0.28 mm. With one or two layers the results showed great divergences from the theoretically expected. This circumstance is to be ascribed to the influence of "unstirred layers" adhering to the membrane surfaces. These extra diffusion layers are not convection proof. By means of a special method their thickness is estimated to be approximately 0.03 mm. The cellophane may have a slight influence upon the mobilities of the ions here investigated which, however, will be accounted for when comparing the results with the theory.

*Choice of Electrolytes*—This was dictated partly by a desire to produce pronounced distribution differences, partly by attention to available analytical methods. Therefore, the first experiment to be reported was carried out with an acid as a diffusing agent and with  $\text{NH}_4\text{Cl}$  as source of the passive ions ( $u_{\text{H}}/v_{\text{ClO}_4}$  being large (cf. p. 110).  $\text{H}_4\text{N}$  and  $\text{Cl}$  are easily determined. In the second experiment a neutral salt, K iodate, was used as a diffusing agent, because the iodate ions have a rather low mobility compared with K ( $u_{\text{K}} = 65$ ,  $v_{\text{IO}_3} = 33$ ). Hydrochloric acid furnished the passive ions.

*Analyses*—The analyses were performed with micro methods. The samples taken had a volume ranging from 0.05 to 1 cc depending upon the circumstances. H was determined by titration with 0.01 N NaOH using methyl red as indicator. Cl was determined electrometrically after the acidity titration, employing 0.005 N AgNO<sub>3</sub> and sulfuric acid. NH<sub>4</sub> was analyzed iodometrically with 0.05 N NaBrO

TABLE I

*Development of Ionic Accumulation and Impoverishment in a System Subjected to a Steady Diffusion*

*Experiment 1*

Initial state

(10 cc)	0.1 N HClO <sub>4</sub>	0.1 N H <sub>4</sub> NCl (1500 cc)
	0.1 N H <sub>4</sub> NCl	
	Inside (i)	Outside (o)
(Membrane)		

H, maintained constant by continuous addition of 5 N HClO<sub>4</sub>

Time	Inside (i)				Outside (o)				(H <sub>4</sub> N) <sub>i</sub> (H <sub>4</sub> N) <sub>o</sub>	(Cl) <sub>o</sub> (Cl) <sub>i</sub>
	H	H <sub>4</sub> N	Cl	ClO <sub>4</sub> *	H	H <sub>4</sub> N	Cl	ClO <sub>4</sub> *		
hrs	mm / l	mm / l	mm / l	mm / l	mm / l	mm / l	mm / l	mm / l		
0	90.9	99.1*	99.1	90.9	0	99.9*	99.9	0	1.00	1.01
0.5	84.1	111	89.0	106	—	—	—	—	1.11*	1.12
2	96.5	159	62.1	193	2.95	99.9	100.9	2.0	1.59	1.62
4	99.6	188	51.6	236	4.31	99.1	101.8	(1.6)	1.90	1.97
6	99.6	207	48.6	258	5.85	102	102	5.9	2.03	2.10
7	96.5	212	48.6	260	—	—	—	—	2.08*	2.10*
8	111	215	47.9	278	8.00	102	101.4	8.6	2.11	2.12
9	105	214	48.6	271	8.40	101	101.5	7.9	2.13	2.09
9 <sup>00</sup>	Potential (i)-(o) Calomel electrodes + 21.0 mv, Ag/AgCl - 0.8 mv †									
20	121	214	49.1	286	17.7	101	100	18.7	2.13	2.04
20 <sup>30</sup>	Potential (i)-(o) Calomel electrodes + 20.2 mv, Ag/AgCl - 1.2 mv †									

\* Calculated figures

† Sign outside

and 0.01 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> according to (13). IO<sub>3</sub> was titrated as I<sub>2</sub> with thiosulfate after addition of KI and acid. Other concentrations were calculated by difference. All pipettes, burettes, and solutions were calibrated against standard solutions. No analytical error is likely to exceed ± 2 per cent.

*Potential Measurements*—The potential measurements were performed by means of calomel electrodes, as described elsewhere (11). Supplementary meas-

measurements were made with Ag/AgCl electrodes prepared according to Brown (14). The electrodes were connected either to a push pull vacuum tube voltmeter, or to a Leeds Northrup Type K potentiometer. The potential figures are regarded as accurate to  $\pm 0.5$  mv.

TABLE II

*Development of Ionic Accumulation and Impoverishment in a System Subjected to a Steady Diffusion*

*Experiment 2*

Initial state  
(10 cc.) ca. 0.4 N KIO<sub>3</sub> | 0.01 N HCl (500 cc.)  
saturated plus  
crystals  
Inside (s) | Outside (o)  
(Membrane)

Time hrs	Inside (I)				Outside (o)				$\frac{(H)_t}{(H)_i}$	$\frac{(Cl)_t}{(Cl)_i}$
	H mm/l	K† mm/l	Cl mm/l	IO <sub>3</sub> mm/l	H mm/l	K† mm/l	Cl mm/l	IO <sub>3</sub> mm/l		
0	0	—	—	—	10.0	—	—	—	—	—
0.1	1.7	371	0.9	372	10.0	0.4	10.0	0.4	0.17	11.1
0.5	10.3	—	—	336	10.0	1.6	10.5	1.1	1.03	—
1	21.0	327	2.9	345	9.8	1.7	9.7	1.8	2.14	3.34
2	37.4	333	4.0	366	—	—	—	2.7	(3.82)	—
3	47.5	328	4.6	376	9.2	4.5	9.9	3.8	5.16	2.15
4	56.0	311	5.0	362	10.2	1.2	9.8	1.6	5.50	1.60
5	62.1	318	5.1	375	10.1	2.5	9.5	2.5	6.16	1.86
6	63.0	315	5.3	383	9.8	4.0	10.1	3.8	6.44	1.90
7	63.0	303	5.2	366	10.9	1.9	10.2	2.6	5.78	1.96
9	61.0	271	5.6	326	10.2	3.3	10.3	3.3	5.98	1.84
10	57.1	248	5.6	300†	—	—	—	—	—	—
9 <sup>§</sup>	Potential (s)-(o) Calomel electrodes + 25.5 mv §									

\* The solution was renewed after 3 and 6 hrs, thereby restoring the initial composition.

† Calculated values.

‡ The decrease in KIO<sub>3</sub> concentration after 6 hrs was probably due to a fall of the room temperature, hence decrease of solubility.

§ Sign outside.

The pH and Cl activity measurements were made by means of a glass electrode (type MacInnes and Dole) (18), and a Ag/AgCl electrode (Brown (14)) respectively using a saturated calomel electrode as reference. A Compton electrometer served as zero instrument.

## RESULTS AND DISCUSSION

In Tables I and II two typical experiments are recorded (Experiments 1 and 2)

*Experiment 1*—This was designed to fulfill, as completely as possible, the main assumptions on which the theory was based (a) Uni-univalent electrolytes being present only, all known as strong and free from complicating phenomena, (b) no migration of water across the convection-proof boundary Perchloric acid served as diffusing agent and the passive ions were  $H_4N$  and  $Cl$  The  $HClO_4$  concentration in the small inside chamber was maintained at about 1/10 molarity by means of an external supply The experiment was started with equal concentration of  $H_4N$  and  $Cl$  ions inside and outside During the following time, however, one could observe that the  $H_4N$  ions moved inwards and, simultaneously,  $Cl$  ions moved outwards Finally a steady state was approached inside, resulting in a 2.13 fold accumulation of  $H_4N$  and a  $Cl$  impoverishment of almost the same magnitude Fig 2 corresponds very closely with the conditions of this experiment No appreciable water migration could be detected

The experimental results will now be compared with a theory  
The theoretical distribution ratio

$$\xi = \frac{(\text{Passive cation concentration})_i}{(\text{Passive cation concentration})_o} = \frac{(\text{passive anion concentration})_o}{(\text{passive anion concentration})_i}$$

can be calculated from either of Equations 1 or 2

According to Equation 1, at  $20^\circ C$  and with the diffusion potential expressed in millivolts,

$$\pi = 58.1 \log \xi \quad (3)$$

In the experiment the membrane potential  $\pi$ , was + 20.2 mv, hence, the distribution ratio is

$$\xi = 2.23, \text{ calculated from observed } \pi$$

(Observed value for  $H_4N$  is 2.13, for  $Cl$  is 2.04 after 20 hours)

According to Equation 2 combined with the relation  $\xi = \frac{(H_4N)_i}{(H_4N)_o}$

$$\log \xi = \frac{u_H - v_{ClO_4}}{u_H + v_{ClO_4}} \log \frac{(H)_i + \xi (H_4N)_o}{(H)_o + (H_4N)_o} \quad (4)$$

$$\text{or} \quad \log \xi = q \log \frac{121 + \xi \cdot 101}{177 + 101} \quad (4a)$$

where  $q$  stands for  $(u - v)/(u + v)$ . There are two possibilities of choosing  $q$ , either to use  $q$  as valid for "free" water, or, to use  $q$  as determined for cellophane. Employing the mobilities  $u_H = 315$  and  $u_{ClO_4} = 64$  as given for free water in the Landolt Börnstein Tables (15),  $q$  becomes 0.66. The relative mobility in cellophane was determined from measurements of the diffusion potentials between 0.1 N and 0.01 N  $HClO_4$  by means of a procedure described elsewhere (11). The mobility ratio,  $u/v$ , thus obtained was 7.55<sup>1</sup> and  $q$  for cellophane was calculated to 0.767. Alternately inserting these  $q$  values in the Equation 4a, the following values of  $\xi$  are obtained

$$\begin{array}{ll} \xi = 1.89 & \text{calculated from Equation 4 when } q = 0.66 \text{ (H}_2\text{O)} \\ \xi = 2.30 & \text{" " " " " } q = 0.767 \text{ (cellophane)} \end{array}$$

*Corrections for Activity Changes*—All discussions so far have been carried out on the supposition that the activity coefficients,  $\gamma$ , were all 1.00. Of course, this is not true when dealing with rather concentrated solutions as is the case in these experiments. Perhaps it may be somewhat meaningless to attempt any adjustments of the experimental results with regard to the activities, because the activity concept was not employed in the theory to be tested. Nevertheless, it may be interesting to introduce some activity corrections. Although, the procedure to be used is only approximate, one should get an idea as to the direction in which the results are influenced by the interionic forces.

The observed distribution ratios obtained from *concentration* figures may be transformed into ratios between activities by multiplying through with appropriate coefficients. Attention will be paid only to the  $H_4N$  distribution. The  $H_4N$  ions were paired on both sides of the membrane with chloride or perchlorate. It seems justifiable to assume that the mean activity coefficients of the two salts are rather similar to those of KCl. Secondly, the usual supposition is made,

<sup>1</sup>  $u/v$  for free water is  $315/64 = 4.94$ , hence the relative cation mobility is about 50 per cent higher in cellophane. For several chlorides the corresponding increase was determined as about 40 per cent (Teorell (11)). A similar relation was observed also for  $KIO_3$  (see footnote 4).

that  $\gamma$  for an electrolyte component in a mixture is the same as in a solution containing only this electrolyte at an ionic strength corresponding to that of the mixture (the rule of Lewis and Randall, *cf* for instance Falkenhagen (16), p. 71). Thus  $\gamma$  for the approximate 0.3 N inside solution was taken as 0.67, and for the approximate 0.1 N outside as 0.75 (according to figures for KCl given by MacInnes and Noyes (17)). Introducing these values, we obtain

$$\text{Observed } \xi \text{ corrected for activity} = \begin{pmatrix} 0.67 & 2.13 \\ 0.75 & \end{pmatrix} = 1.91$$

Corresponding activity corrections may also be applied to Equation 4 *a*, ( $\gamma$  for  $\text{HClO}_4$  was taken as 0.78 and 0.82 inside and outside respectively) The results are

$\xi = 1.54$ , calculated from Equation 4 and corrected for activity when  $q = 0.66$  ( $H_2O$ )  
 $\xi = 1.70$ , "  
 lophane)  $q = 0.767$  (cel-

*Experiment 2* —The experimental conditions were not quite ideal, because certain ionic interactions took place, and water migration also occurred. These factors were not considered in the theory. Therefore, this experiment is an interesting one, giving some information as to the sensitiveness of the proposed theory towards new factors. The diffusing agent was potassium iodate, being in a saturated solution inside and kept in contact with an excess of  $\text{KIO}_3$  crystals. The passive ions were formed from hydrochloric acid, at the beginning of the experiment being present on the outside only. During the course of the experiment the ionic concentration outside remained practically constant because of its large volume and repeated renewals. In the inside chamber, however, a redistribution of the electrolytes was developed resulting in a steady state. Here, the passive cations, in this case the H ions, were accumulated<sup>2</sup>. The passive anions, the Cl anions, in the final steady state attained a considerably lower concentration inside than outside (Table II).

Qualitatively the observed diffusion effect upon the ionic distribution is in agreement with the theory. With the direction the diffusion

<sup>2</sup> The increase of  $C_H$  inside could be nicely demonstrated, if more dilute HCl was used, by the color changes of an added indicator such as brom phenol blue

potential has, the negative sign being inside, an accumulation of passive cations and impoverishment of anions is to be expected, and was also found. As to the quantitative relations this experiment, at first, seems to show some discrepancies between theory and observations

TABLE III

*Hydrogen Ion Activity Measurements of Inside and Outside Solutions after 9 Hours*  
*Experiment 2*

(P.D. measured with glass electrode/calomel electrode, slope" 57.5 mv)

Solution	Total H concentration	Millivolts	pH	$a_H$
	<i>mM/l</i>			<i>mM/l</i>
Inside	61.0	-18.6	1.61	38.9
Outside	10.2	+5.1	2.02	9.55
0.01 N HCl (control)	10.0	+5.6	2.03	9.34
(i)-(o)	50.8	-23.7	-0.41	$\frac{(a_H)_i}{(a_H)_o} = 2.57$

TABLE IV

*Chloride Ion Activity Measurements of Inside and Outside Solutions after 9 Hours*  
*Experiment 2*

(P.D. measured with Ag/AgCl electrode/calomel electrode, "slope" 57.7 mv)

Solution	Total Cl concentration	Millivolts	pCl	$a_{Cl}$
	<i>mM/l</i>			<i>mM/l</i>
Inside	5.6	-118.8	2.37	4.20
Outside	10.3	-96.5	1.98	10.2
0.01 N HCl (control)	10.0	-98.9	2.03	9.34
(i)-(o)	-4.7	-22.3	0.39	$\frac{(a_{Cl})_i}{(a_{Cl})_o} = 2.43$

However, when due attention is paid to certain interfering circumstances, the results also appear to agree quantitatively in a satisfactory manner with the theory. For this purpose the following discussion is given.

First, in Table II it will be noticed that the final distribution ratio



$H_i/H_o$  is as high as 5.98, the ratio  $Cl_o/Cl_i$ , however, is much lower, 1.84. The  $H$  figures were determined by titration, accordingly all hydrogen ions, both free and bound, were included. It is well known that  $IO_3^-$  ions can form  $H(IO_3)_2$ , bi-iodate, in *acid* solution, thus binding hydrogen ions. Unfortunately, pH measurements were not performed during the actual run, but afterwards "artificial" inside and outside solutions were made up to correspond with the analytical figures recorded in Table II at 9 hours.<sup>3</sup> In these solutions pH and  $a_{Cl}$  were determined by means of the glass electrode and AgCl electrode respectively. The results are summarized in Tables III and IV. It can be seen that the  $H$  ion activity inside is considerably lower than the total  $H$  concentration, as pointed out, probably due to formation of bi-iodate ions. From these measurements the ionic distribution ratios, expressed in activity figures, will be found as

$$\begin{array}{l} \text{Observed } \xi = 2.57 (5.98), \text{ corrected for } H \text{ activity} \\ \text{" } \xi = 2.43 (1.84), \text{ " " } Cl \text{ " } \end{array}$$

The values within brackets are the ones obtained from total concentration figures.

Hence, the relation  $H_i/H_o = Cl_o/Cl_i$  seems to be obeyed, as demanded by the theory. The theoretical  $\xi$  values are calculated, as in Experiment 1, either from the observed diffusion potential (Equation 1), or from the transcendental formula (Equation 2). For the first alternative is obtained

$$\xi = 2.75, \text{ calculated from the diffusion potential}$$

Calculations on the basis of Equation 2 can be performed in several different ways. First, there are two choices of the relation  $u/v$  or of  $q$ , referring to free water or to cellophane. Secondly, when dealing with a rather concentrated salt solution (saturated  $KIO_3$ ), some kind of correction for activity changes may be demanded. Based on concentration figures given for 9 hours in Table II it is found that

<sup>3</sup> These "artificial" solutions were made *inside*, 5.6 cc 0.1 N HCl, 55.4 cc 0.1 N  $HIO_3$ , 5.79 gm  $KIO_3$  *outside*, 10.2 cc 0.1 N HCl, 0.1 cc 0.1 N KCl, 3.2 cc 0.1 N  $KIO_3$ , in all cases  $H_2O$  was added to make 100 cc of solution. The reagents were Kahlbaum brands, the solutions were freshly made and standardized.

$\xi = 2.86$ , calculated from Equation 2 when  $q = 0.327$  ( $\text{H}_2\text{O}$ )<sup>4</sup>  
 $\xi = 5.14$ , " " " " " " " " when  $q = 0.509$  (cellophane)

**Corrections for Activity Changes**—When attempting activity corrections, it was assumed that the mean activity coefficient,  $\gamma$ , for the K-iodate inside was the same as for 0.3N KCl being 0.67.  $\gamma_{\text{HCl}}$  outside was taken as 0.93, a figure valid for 0.01N HCl (according to MacInnes and Noyes). Any further corrections do not have any appreciable effect upon the results and must also be rather ambiguous. Therefore they are omitted. The  $\xi$  values obtained from activity figures were

$\xi = 2.55$  calculated from Equation 2 and corrected for activity when  $q = 0.327$  ( $\text{H}_2\text{O}$ )  
 $\xi = 4.25$  " " " " " " " " " " " " " " when  $q = 0.509$  (cellophane)

TABLE V  
 Comparison between Observed and Theoretical Distribution Ratios ( $\xi$ ) of Passive Ions in Steady State

$$\xi = \frac{(\text{Passive cation})_{\text{inside}}}{(\text{Passive cation})_{\text{outside}}} = \frac{(\text{passive anion})_{\text{outside}}}{(\text{passive anion})_{\text{inside}}}$$

Experiment	Diffusing agent	Passive ion	Observed from		Calculated from Equation 2 (4)				Calculated from diffusion potential
			Concentration	Activity	Free water		Cellophane		
					Concentration	Activity	Concentration	Activity	
1	HClO <sub>4</sub>	H <sub>4</sub> N	2.13	1.91	1.89	1.54	2.30	1.70	2.23
		Cl	2.04	(1.87)					
2	KIO <sub>3</sub>	H	5.98	2.57	2.86	2.55	5.14	4.25	2.75
		Cl	1.84	2.43					

#### SUMMARY OF RESULTS AND CONCLUSIONS

In Table V the experimental and theoretical results are put together. The figures seem to justify the following conclusions:

1. The agreement between observed and calculated  $\xi$  values is, on the whole, quite satisfactory in both the experiments, if proper attention is paid to activity conditions.  $\xi$  as calculated from the transcendental

<sup>4</sup> Calculated value from  $\mu_{\text{K}}/\nu_{\text{IO}_3}$  determined from diffusion potential across cellophane of 0.1 – 0.01N  $\text{KIO}_3$  according to (11). This value was found to be 3.08 (in free water 65/33 = 1.98).

Equations 2 or 4, using the ionic mobilities for free water, yields the best agreement

2 If the mobility relations as determined for cellophane are used in the theoretical calculations, much higher  $\xi$  values than those actually observed would be expected in the  $\text{KIO}_3$  experiment. In the  $\text{HClO}_4$  experiment it is hard to point out any significant difference as to the outcome of different choice of mobility relations. Hence, it appears that it would be better to assume a validity of free water ionic mobilities, in spite of the fact that the diffusion effects occurred across cellophane. Such a conclusion also follows from the views expressed by the author, that the cellophane mobility figures as determined are only *apparent* values. The reason is to be sought in interference from the membrane itself (Teorell (12))

3 The theoretically expected  $\xi$  values when calculated from the *measured* diffusion potential are of the same magnitudes as the observed figures corrected for activity. The latter are only 14 and 9 per cent lower than the former, in Experiments 1 and 2 respectively, these differences may very likely arise from small, unavoidable liquid junction potentials at the calomel electrodes. This fact seems to offer an ample confirmation of one consequence of the theory, namely, that the distribution of the passive ions is completely determined by the *observed* diffusion or membrane potential according to the simple Equation 1, or

$$\log \xi = \frac{\pi}{0.1983 T} \quad (1)$$

( $\pi$  = millivolts,  $T$  = absolute temperature)

In regard to the thermodynamical interpretation of this equation we may refer to the first paper in this series (1), paragraphs IVb and c

4 The agreement between experiment and theory according to Equation 1 is of a profound and essential significance. This shows that the water migration does not have any appreciable influence upon the expected ionic distribution, although amounting to about 40 per cent as volume increase inside in Experiment 2. Furthermore, the high  $\xi$  values as calculated from cellophane mobilities in Experiment 2 can be ruled out as being improbable, because such figures would correspond to a value of the diffusion potential much higher than that

actually observed. This point offers additional evidence for the views expressed under paragraph 2 above.

5 Still, there are some other points which can be advanced giving beautiful confirmation of the proposed theory. In the theoretical paper (1), in paragraph IVc it was remarked

As the diffusion potential  $\pi$  is equal to the expression for the electrode potential for any passive ion, a concentration chain consisting of reversible electrodes for such an ion species placed in the solutions (o) and (i) should give no current.

In Experiment 1 the actual "concentration chain potential" with AgCl electrodes, reversible in regard to the passive chloride ions, was measured from  $-0.8$  to  $-1.2$  mv. The theoretical value should be  $\pm 0$  mv, accordingly the result is not far outside the experimental limit of error. At the same time  $\pi$  was about  $+21$  mv (cf Table I).

In Experiment 2 the same thing can be shown somewhat more in directly. Here reversible electrodes for both the passive ion species were available, the glass electrode for the H ions, and the AgCl electrode for the Cl ions. The "electrode potentials" for these two ion species are simply found as differences between the potential values recorded in Tables III and IV, as measured against the same reference (calomel) electrode. These values are (cf also the tables),

Electrode potentials	$\left\{ \begin{array}{l} [(mv)_i - (mv)_o]_{\text{glass}/H^+Cl^-} \\ [(mv)_i - (mv)_o]_{\text{AgCl}/H^+Cl^-} \end{array} \right.$	$-23.7 \text{ mv}$
		$-22.3 \text{ mv}$
Diffusion potential observed $\pi$		$+25.5 \text{ mv}$

It is noticed that the figure for the diffusion potential is almost equal to the figures for the practically identical electrode potentials. As it carries opposite sign, the resulting concentration chain potentials, being the sum of both (diffusion potential + electrode potential), also in this case, will not be far from zero.

#### SUMMARY

The aim of this paper is to present confirmation of the theory of the diffusion effect. By diffusion effect is understood a redistribution of the ions in a system where a continuous diffusion of one electrolyte is going on, which results in a steady state showing accumulation and impoverishment of certain ions.

Some typical experiments are recorded and discussed. The results are found in satisfactory agreement with the theory; this can be demonstrated in several ways.

The importance of considering activity changes is pointed out. Water migration and the diffusion membrane probably have no appreciable influence upon the diffusion effect under the conditions of these experiments.

The diffusion effect may have biological analogies.

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# THE EFFECT OF AUXINS UPON PROTOPLASMIC STREAMING

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## INTRODUCTION

Although the existence of growth substances, or auxins, which produce cell enlargement in plants has been known for some time, the mechanism by which they exert their effect is still little understood. A full account of this field is given in Went and Thimann (1937). Briefly, it has been shown within the last 4 years that auxins cause not only cell enlargement but also bring about root formation, inhibition of bud development, inhibition of root elongation, and other effects, this multiplicity of activities indicates that the auxins must exert some fundamental action upon the plant cell. Even in cell enlargement the action must be of a very deep seated nature, for while Heyn and Soding have shown that the action of auxin results in an increase of cell wall plasticity, this does not appear to result from a direct reaction between auxin and cell wall material. Thus the results of Thimann and Bonner (1933) show that there is no stoichiometrical relation between the amount of auxin entering and the amount of cellulose or of total new wall material deposited as a result, nor is it possible for the auxin to produce a monomolecular layer upon the new cell wall. The action on the cell wall must therefore be realized in some indirect way, and is probably the result of a chain of reactions originating inside the cell.

Changes in growth rate are not in general measurable until some considerable time after the application of the auxin. Thus, in measurements of straight growth or of curvature of the *Avena* coleoptile, a period of 15 to 30 minutes usually elapses before the increased growth rate, or the curvature, becomes detectable. The first of the



series of reactions ultimately leading to increased wall plasticity and elongation, or to the other effects mentioned above, presumably begins very much earlier than this, most likely immediately the growth substance has penetrated into the cell. It is therefore desirable to find a method by which quantitative measurement of the *immediate* effect of auxin might be made, in view of the above considerations, such effect must be looked for not in the wall but in the contents of the cell, that is, in the protoplasm.

One of the most obvious properties of the protoplasm is its streaming, which in the *Avena* coleoptile, especially in the epidermal cells, is readily observed and measured. Immediate changes in the streaming have been found by various investigators to take place as a result of temperature change (Ewart, 1903, Bottelier, 1934), visible light (Bottelier, 1934), x-rays (Williams, 1923, 1925), and miscellaneous dissolved substances (Ewart, 1903). In an investigation with Prof. I. W. Bailey of the effect of auxin on cambium cells, one of us (K. V. T.) observed that streaming was very rapid in the preparations treated with auxin. There was also a change in the extent of the streaming, the moving particles being carried up to the extreme tips of the cells, which previously were stagnant. It was therefore decided to make a study of the protoplasmic streaming in favorable material, in order to determine whether auxin exerted any effect upon it and whether the rate of streaming could be used as a measure of the immediate physiological changes brought about by auxin within the cell.

### *Materials*

Indole-3-acetic acid, the most active auxin readily available in pure state, was used for the bulk of the experiments. The sample used was prepared and purified more than 2 years ago (Thimann and Koepfli, 1935) and its activity is the same as when first prepared. For coumaryl-3-acetic acid the authors are indebted to Prof. T. Reichstein of Zurich, for pure allocinnamic acid to Prof. A. J. Haagen-Smit. These substances were used in concentrations ranging from 0.005 up to 10 mg. per liter, which include all those causing physiological response.

The water used for all solutions and in controls was redistilled in a pyrex still and stored in paraffined glass containers.

The Cornellian strain of oats was used, its properties, growth rate, dimensions, etc., are essentially the same as those of "Segershavre" used in other experiments.

### Methods

The seeds were husked, soaked for 2-4 hours, germinated on moist filter paper in a dark room with occasional red light, and planted in moist sand at 24 and 85 per cent relative humidity. The coleoptiles were slit lengthwise, removed from the plant, and spread out upon a slide with the cut face down, as described by Bottelier (1934). The slight pressure of the cover slip was sufficient to hold them in place. The zone studied was about 1 cm below the tip. In this series of experiments coleoptiles 5 to 6 days old from the time of soaking were used; these measured 4 to 5 cm in length. The effect of auxins on younger coleoptiles will be discussed in a later paper.

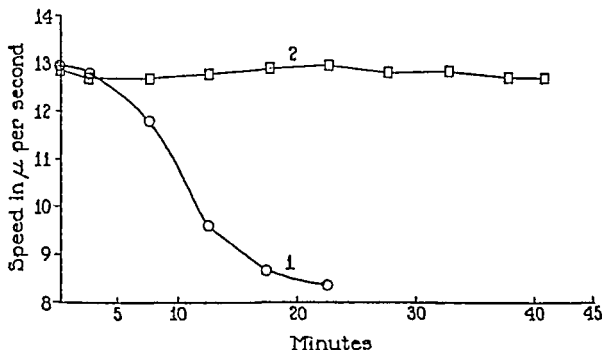


FIG 1 Rate of streaming of epidermal cells of *Avena* coleoptiles in pure water. Curve 1, not aerated, curve 2, aerated, (mean of five experiments)

After cutting, the coleoptiles were allowed to recover in water for 5 minutes. Continuous readings were then taken for 5 minutes in water after which the water was removed from under one side of the cover slip with filter paper and the solution to be tested was added to the other side. Further solution was added at frequent intervals during the experiment. Continuous readings were made of the motion of the smallest visible particles in a single epidermal cell, using a calibrated ocular micrometer and a stop-watch. The mean of about ten readings within a 3 minute period was taken to establish the streaming rate for that period.

Since Bottelier (1934) has shown that the streaming in *Avena* is sensitive to light, all readings were made in the dark room by red light, heating of the slide being avoided by the interposition of a water cell about 5 cm thick.

### *Influence of Aeration*

When the coleoptiles were mounted as described, in pure water, the rate of streaming showed a steady decrease (Fig 1, curve 1), which became apparent in from 2 to 5 minutes Bottelier (1935) noted a similar decrease, and found that it disappeared if a stream of fresh solution was passed under the cover slip He attributed this fall in the rate of streaming to the rapidly diminishing amount of oxygen in the solution under the cover slip, and showed that oxygen is one of the principal factors controlling the streaming rate That the cause of the decrease in rate in our experiments is oxygen deficiency is confirmed by the fact that no such decrease took place when aerated water was used (Fig 1, curve 2) Under these conditions, the streaming rate, as can be seen, remained constant for at least an hour Aeration was accomplished by bubbling air through the water for 20 to 30 minutes immediately before using it All control experiments were therefore made with water thus aerated, and solutions to be tested were made up with the same water

### *Qualitative Effect of Auxin*

Dilute solutions of indole-3-acetic acid (0.005 to 0.5 mg per liter) induced an increase in the rate of streaming, the extent of which was dependent on the concentration used The rate increased from 13 to about 18  $\mu$  per second when the coleoptiles were treated with a solution containing 0.010 mg per liter, about the optimum concentration (Fig 2) The effect of immersing coleoptiles in the auxin solutions began to appear immediately, the increase in rate being observed well within the first 5 minutes of treatment The maximum streaming rate was obtained at 10 to 15 minutes' exposure, after which the rate slowly fell again to reach that in water at 30 to 40 minutes from the time of application of the solution This time relation was observed in all the experiments with auxin of any kind In no case was the effect latent, nor was the maximum rate of streaming found earlier or later, and never was the effect found to last beyond 30 to 40 minutes Finally, the streaming always returned to exactly the same rate as before auxin was applied

When relatively concentrated solutions of indole-3-acetic acid were used (above 0.5 mg per liter), the effect was to decrease the rate of

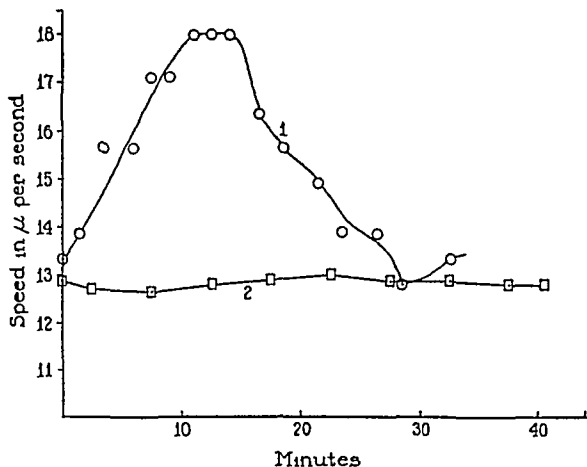


FIG 2 Typical effect of low concentrations of auxin on the rate of streaming  
Curve 1, 0.007 mg indole 3 acetic acid per liter curve 2, pure water

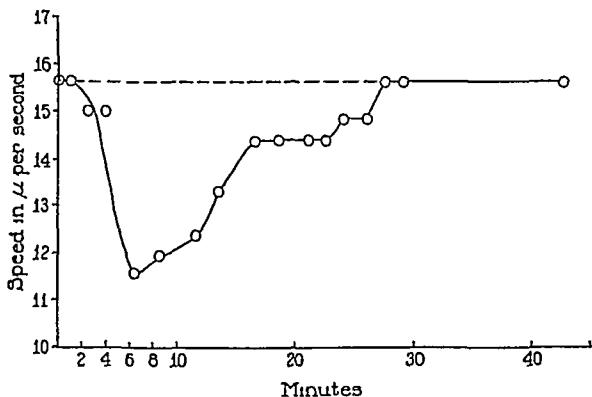


FIG 3 Typical effect of high concentrations of auxin on the rate of streaming  
Curve 1, 5 mg indole 3 acetic acid per liter

streaming The curve thus obtained (Fig 3) is the reverse of that of Fig 2 The effect of high concentrations also takes place very rapidly, beginning in 2 to 5 minutes, reaching its maximum in 5 to 20 minutes, and returning to the rate in water at about 30 minutes

### *The Quantitative Effect and Its Relation to Concentration*

In the curves of rate against time, such as those in Figs 2 and 3, a quantitative measure of the total effect of a given auxin concentration is evidently given by the *area* under the curve The value thus obtained combines the maximum speed reached and the duration of the effect It is expressed in units essentially of length, since it is a product of a velocity by a time ( $LT^{-1} \times T = L$ ) Clearly this value represents the total extra distance travelled by one particle when it is acted upon by auxin Since the effect of auxin on growth is also expressed in units of length (at least for coleoptiles), a comparison may justifiably be made between the two effects

Each experiment, comprising 20 or 30 average velocities determined over a 40 minute period, thus gives a single value for the total effect of auxin at the concentration used Since the values obtained from different experiments at the same concentration agree fairly well, the mean of several such values represents an accurate measure of the effect It is to be noted that each final value for the effect of a given concentration is therefore founded on about 1000 actual velocity readings Experiments were made in all at thirteen different auxin concentrations

The mean final values are plotted in Fig 4 against the logarithm of the auxin concentration It may be seen that the effect, that is, the mean extra distance travelled by a given particle increases linearly with concentration from zero at 0.0025 mg per liter to a maximum at 0.01 mg per liter From this point, the effect decreases with further increase in concentration to reach zero at about 0.5 mg per liter, and negative values at still higher concentrations The curve thus consists essentially of two straight lines separated by an intermediate region around the maximum

It is of interest to compare the effect of auxin on streaming with its effect on growth Measurements of elongation of coleoptiles immersed in solution are obviously the most nearly comparable with the data on

streaming Such measurements, using short sections from coleoptiles, were first made by Bonner (1933) and have since been used by several workers Jost and Reiss (1936, 1937), using longer sections dipping in solution only at one end, found that the maximum growth reaction in 24 hour tests took place at 10 mg per liter Higher concentrations gave less growth, though still more than controls in pure water In order to have comparable data we have made measurements on 3 mm

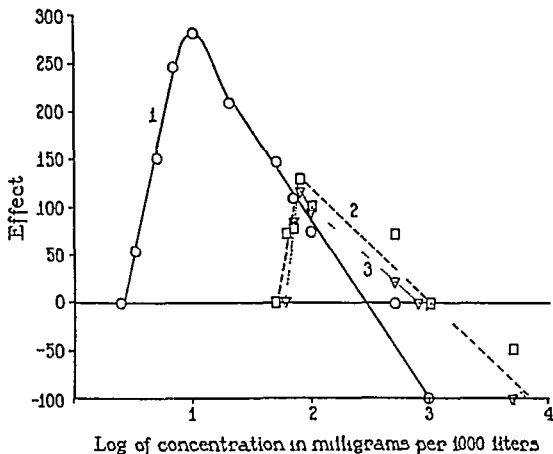


FIG. 4 Total effect of auxin on the rate of streaming each point derived from the mean of several curves of the type of Figs 2 and 3 Curve 1 indole 3 acetic acid curve 2, allocinnamic acid curve 3, coumaryl 3 acetic acid The ordinates represent the effect in change of velocity over that of controls  $\times$  time

sections of 5 day old coleoptiles, immersed in shallow layers of solutions as described by Bonner (1933), using the zone about 1 cm below the tip

Fig 5 summarizes the data from two such experiments, using coleoptiles with the primary leaf just broken through and protruding about 5 mm The type of curve obtained is clearly the same as that obtained with streaming, and it is of interest to note that very high

auxin concentrations may cause an actual shrinkage of the coleoptiles, although the tissue appears normal and shows no increase in width. The peak of the growth effect is obtained at 10 mg per liter or close to

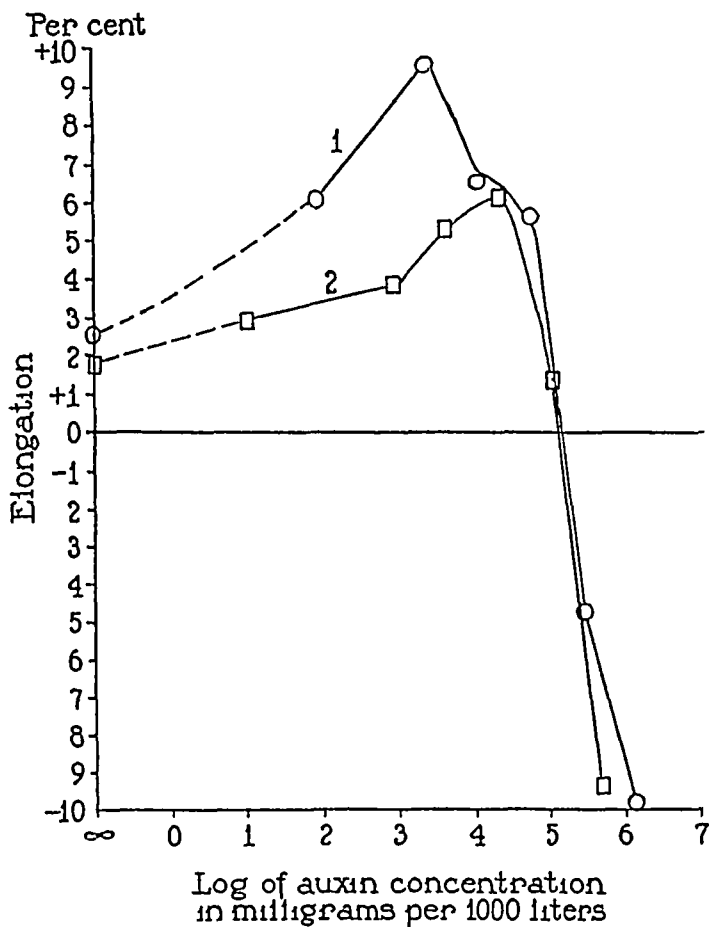


FIG 5 Effect of auxin on the elongation of sections of coleoptiles immersed in solution. Abscissae, logarithm of the auxin concentration as in Fig 4. Ordinates, elongation in per cent of original length after 24 hours' immersion. The abscissa marked  $\infty$  refers to controls in water. Curves 1 and 2 represent two separate experiments, each point being the mean of 15-25 sections in each solution.

it, agreeing with the result of Jost and Reiss, while the maximum effect on streaming occurs at 0.01 mg per liter. The streaming curve is then displaced to the left of the growth curve by about 3 logarithmic units. The significance of this displacement is not clear.

*Specificity of the Auxin Effect*

The question now arises whether this phenomenon of increase in the rate of streaming is a specific one for growth substances. In order to determine this, experiments of two kinds were made (a) the effects of other auxins on streaming were studied, using the procedure as above, and (b) other substances, not growth promoting, but previously known to affect streaming of protoplasm in various materials, were studied for their effect under our conditions.

(a) Coumaryl-2 acetic acid and allocinnamic acid were found to be active as growth promoting substances by Thimann (1935) and Haagen Smit and Went (1935) respectively. Instead of the former we have used for these experiments coumaryl 3 acetic acid, i.e. the oxygen analog of indole 3 acetic acid, its activity is somewhat higher than that of the 2 substituted derivative. Both coumaryl 3 acetic and allocinnamic acids have an activity on growth of immersed coleoptile sections of from a tenth to one hundredth of that of indole 3 acetic acid. Study of their activity on streaming showed that they both accelerate the rate at low concentrations. Plotting the total effect against concentration (Fig 4, curves 2 and 3) shows that their activity reaches a peak at a concentration ten times the optimum concentration of indole 3 acetic acid. The curves for allocinnamic and coumaryl 3 acetic acids fall very close to one another and are approximately parallel to that for indole 3 acetic acid. The total increase in rate is, however, less than that caused by the latter. When it is borne in mind that both of these substances are what could be described as auxins of only moderate activity, it will be seen that their effect on streaming is qualitatively and quantitatively about what would be expected if this effect is related to their activity as auxins.

(b) Ethylene chlorhydrin has no auxin activity, although it has the effect of breaking dormancy of certain tubers (Denny, 1926, 1934). The streaming of the protoplasm of *Elodea* and *Nitella* was found by one of us (Marcy, 1937) to be stimulated by concentrations of 0.25 to 0.075 gm ethylene chlorhydrin per liter, after immersion of the plant material in the solution for 24 hours. In the experiments here described, concentrations from 0.1 to 0.0001 gm per liter were tested, and all failed to show any effect on streaming in *Avena* coleoptiles within the 30 to 40 minutes required to reach the total effect of indole



3-acetic acid, coumaryl-3-acetic acid, or allocinnamic acid. The average of the results of three experiments at 0.01 gm per liter is shown in Fig. 6, curve 1.

The extensive experiments of Fitting (1929, 1932) showed that  $\alpha$ -amino acids, among them histidine, cause the renewal of active streaming in leaf cells of *Vallisneria*, in which streaming has previously been stopped by placing the plants in glass-distilled water. Histidine was found to be active in concentrations of one part in  $80 \times 10^6$  or

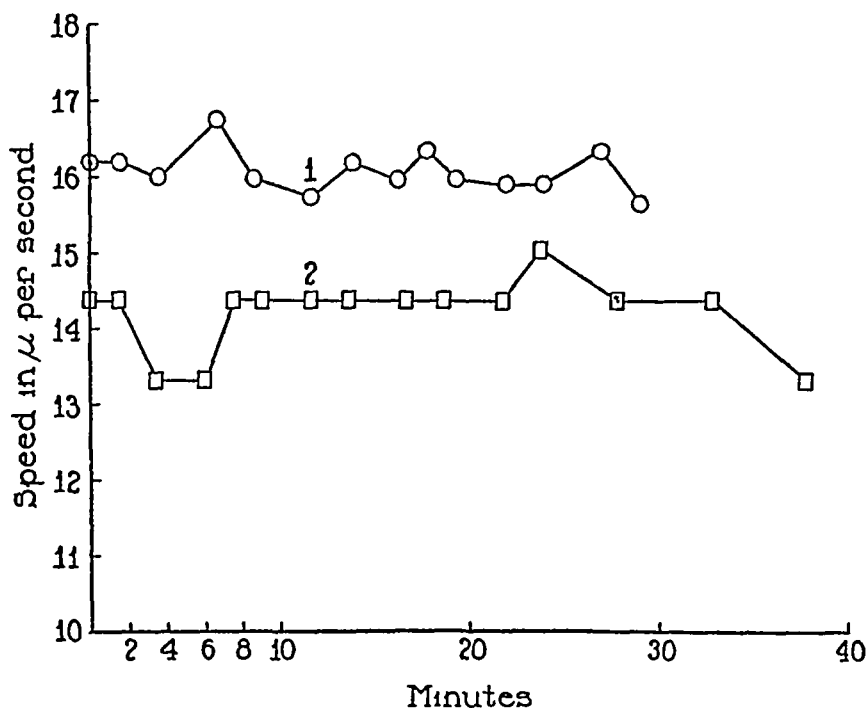


FIG. 6. Curve 1, the effect on the rate of streaming of ethylene chlorhydrin, 10 mg per liter, (mean of three experiments). Curve 2, effect of histidine, 0.02 mg per liter.

0.0000001 M. Histidine was therefore tested in our experiments at concentrations of 0.005, 0.01, 0.05, and 0.1 mg per liter. In no experiment did it have any effect on the rate of streaming in *Avena*. The results of one experiment are plotted in Fig. 6, curve 2. There was no consistent variation either with concentration or with time, the variations observed being no greater than those in single control experiments in pure water.

Urea, in concentrations of 10 and 0.1 mg per liter, also was without effect on streaming in *Avena* coleoptiles.

Since the auxins used above are all weak acids the possibility remains that their effects on streaming might be due to changes in pH. The pH of the stock solution (100 mg per liter) was found colorimetrically to be about 6.0. Now the maximum effect of indole 3 acetic acid was obtained in solutions containing 0.01 mg per liter, *i.e.*,  $m/10^7$ . Even if the acid were as strong as hydrochloric acid, the pH of this solution would be, by definition, 7.0. However, dilute solutions of acetic acid were tested for the sake of completeness and found to have no effect on streaming either at 0.001 M or at 0.000001 M (Fig. 7).

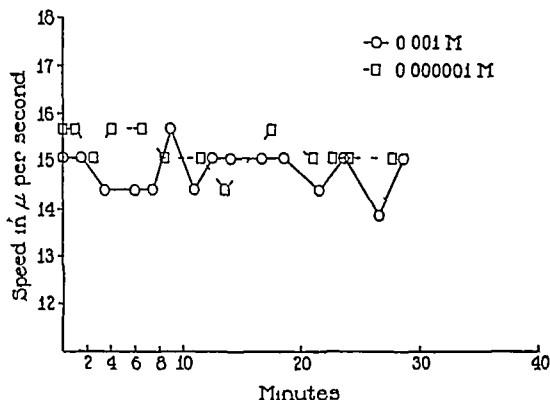


FIG. 7 Effect of acetic acid on the rate of streaming

#### DISCUSSION AND SUMMARY

1 Evidence has accumulated that the action of auxins in promoting growth is exerted not upon the cell wall but upon the cell contents, *i.e.*, the protoplasm. Following indications previously obtained, therefore, the effect of auxins on the rate of protoplasm streaming in the *Avena* coleoptile was studied.

2 Indole 3 acetic acid, the most active auxin available in pure form, was found to increase the rate of streaming in the epidermal cells of the *Avena* coleoptile at concentrations between 0.5 and 0.002 mg per

liter, the maximum increase being brought about at 0.01 mg per liter. This concentration is approximately that which, applied in agar to one side of the decapitated coleoptile, would give a curvature of  $1^\circ$ , *i.e.*, it is well within the range of concentrations active in growth promotion. It is, however, much less than that which produces maximum elongation in immersed sections of *Avena* coleoptiles.

3 This accelerating effect is readily determined quantitatively by comparison with the streaming in control coleoptiles in pure water, which, if thoroughly aerated, maintain a constant rate for over an hour. The accelerating effect takes place immediately and is over within about 30 minutes.

4 Concentrations of indole-3-acetic acid greater than 0.5 mg per liter inhibit the streaming, the effect being also over in about 30 minutes, and its extent increasing with increasing auxin concentration. This parallels the effect of high auxin concentrations in inhibiting elongation, although the inhibition of streaming is obtained at much lower concentrations than inhibit elongation.

5 The effects of indole-3-acetic acid on streaming are not specific for that substance, but appear to be common to auxins in general. Thus coumaryl-3-acetic acid and allocinnamic acid, both of which bring about cell enlargement, root formation, and bud inhibition, *i.e.* are typical auxins, also cause an immediate acceleration of the rate of streaming, and as with indole-acetic acid the effect is over in about 30 minutes. The concentrations of these two substances which produce the maximum effect are about ten times that of indole-acetic acid, which approximately corresponds with their relative auxin activities. The curves relating concentrations of these substances to their effects on streaming are very similar to that for indole-acetic acid.

6 On the other hand, certain substances which are known to affect streaming in other materials do not produce any effect comparable to that of auxin. Ethylene chlorhydrin, histidine, and urea in all concentrations were without effect on streaming in the *Avena* coleoptile within the first 30 minutes of treatment.

7 The effects produced by the auxins were not due to pH.

8 The action on streaming here studied is evidently quite different from the re-starting of streaming after its cessation, studied by Fitting in *Vallisneria*. Correspondingly histidine, which in Fitting's experiments showed activity down to  $10^{-7}$  M, is inactive here.

9 *Per contra*, the effect of auxin here studied is on *normal* streaming. It takes place immediately and at concentrations in the same range as those which produce growth. The curve of effect against concentration parallels that for growth although the actual concentration values differ. It is therefore reasonable to suppose that the effect of auxin on streaming is closely connected with one of the first stages of its effect on the growth process.

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# THE RELATIONSHIP BETWEEN BACTERIAL GROWTH AND PHAGE PRODUCTION

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Earlier work on the bacterium bacteriophage reaction (1) has stressed the importance of bacterial growth as a conditioning factor for phage production. It was found that the rate of phage formation could be expressed in terms of the rate of bacterial reproduction and from this single differential equation there were derived integral forms predicting satisfactorily the time of lysis, number of bacteria present when lysis begins, etc. In the basic equation the rate of phage production was expressed as a power of the rate of bacterial reproduction,  $z c$ , in a mixture of phage and growing bacteria the ratio of phage to bacteria continually increases. Finally, when a certain threshold value is attained (100-140 activity units per bacterium) the process of cellular dissolution or lysis begins. Other workers have since reported practically identical kinetic mechanisms for other phages and other bacteria (2-3).

Despite the fact that the available experimental evidence indicated bacterial growth to be the prime conditioning factor for phage production there remained the possibility that under some conditions the two phenomena might be dissociated. This possibility was confirmed by Scribner and Krueger (4) in an investigation of the reaction between phage and susceptible bacteria in the presence of 0.25 molar NaCl. Under these special conditions a prolonged maximal stationary phase of bacterial growth occurred and during this time phage continued to be produced at the usual rate.

We have conducted further experiments dealing with the effect of temperature and pH on the phage bacterium reaction, and have

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found that bacterial growth definitely is not the essential conditioning factor for phage formation

### *Methods*

1 The medium used throughout these experiments was beef infusion broth containing 1 per cent Difco peptone, 0.5 per cent NaCl and was adjusted so that the final pH after sterilization was 7.4

2 The bacteriophage and bacteria were the same ones used in previous studies<sup>1</sup> The phage contained  $1 \times 10^{10}$  activity units/ml The staphylococcal suspensions for each experiment were prepared from eighteen (18) hour cultures grown on nutrient agar in Roux flasks and harvested in broth

3 The quantitative determination of bacteriophage was carried out by the activity method of Krueger (5) The accuracy of the method, which has been used in this laboratory for the past 6 years, is  $\pm 5$  per cent

4 In studying the effects of different temperatures on the phage-bacterium reaction a mixture containing  $2 \times 10^7$  bacteria/ml and  $1 \times 10^7$  phage units/ml in 100 ml of broth at pH 7.4 was placed in the water bath shaker at the desired temperature 5.0 ml samples were withdrawn every 0.3 hour for determining [bacteria] by the turbidity comparison method described in an earlier paper (5) At intervals of 0.4 hour 0.5 ml of the mixture was pipetted into 4.5 ml of iced broth These 1/10 dilutions were kept in ice water until the end of the experiment and were then used for determining [phage]

Controls consisted of phage solutions containing known concentrations of active phage which were exposed to the temperatures used for 3 hour periods No inactivation of phage was detected in any of the solutions

5 To determine the effect of various H-ion concentrations on the phage-bacterium reaction normal NaOH or normal HCl was added to the broth in sufficient quantity to produce the desired change in pH Mixtures of phage and bacteria in 100 ml amounts of the broth were made so that the initial bacterial concentration was  $2 \times 10^7$  cells/ml and the initial phage concentration was  $1 \times 10^7$  activity units/ml pH was determined by the glass electrode method at the beginning and at the end of each experimental period It was found that the broth normally exerted sufficient buffer action to maintain constant pH and no additional buffer mixtures were used The phage-bacteria mixtures were shaken in the water bath at 36°C and samples were taken at intervals for determination of [bacteria] and [phage] as described under paragraph 4 above As controls, to determine the effect of each pH value on phage at 36°C, solutions containing known amounts of phage were adjusted to various H-ion concentrations and were shaken in the water bath for 3 hour periods It was found that there was no detectable inactivation of bacteriophage under these conditions

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<sup>1</sup> Reported for the most part in the *Journal of General Physiology* and the *Proceedings of the Society for Experimental Biology and Medicine* from 1929 on

## RESULTS

1 *The Effect of Temperature on the Phage Bacterium Reaction*

Figs 1, 2, 3, and 4 show the course of bacterial growth and phage production at 30°C, 35°C, 40°C, and 45°C respectively. At 30°C

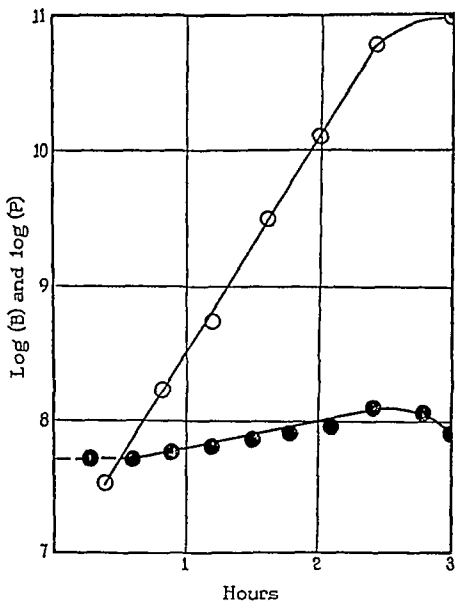


FIG 1

FIGS 1-4 The effect of temperature on the phage bacterium reaction. Logarithms of [bacteria] and [phage] are plotted against time. ○ = phage activity units/ml ● = bacteria/ml. FIG 1 Temperature 30°C

the bacterial population increases 1.5 fold each hour, during the same interval phage concentration increases 40 fold. With a 5° temperature increment the bacterial population shows an increase of 2.5 fold



per hour and the phage concentration a corresponding increase of 160-fold That is, while the rate of bacterial reproduction rises

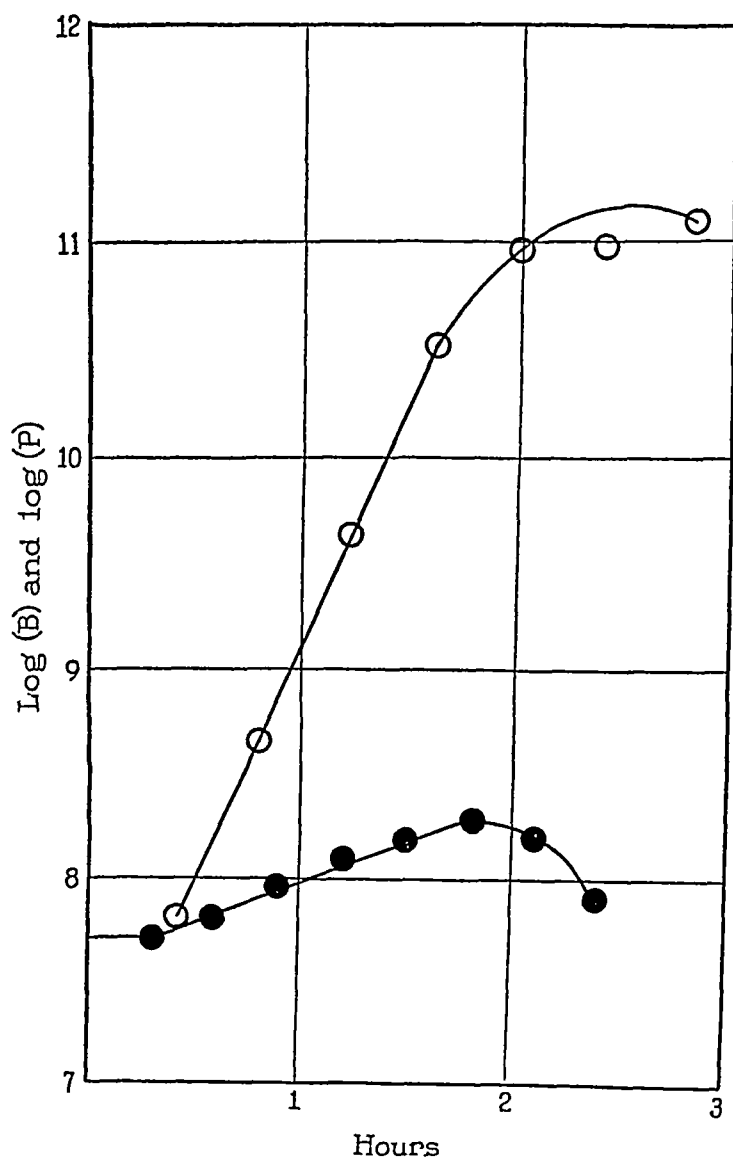


FIG 2 Temperature 35°C

about 1.7 times, the rate of phage production increases 4 times. It is interesting that a further rise in temperature to 40°C brings the rate of bacterial reproduction up to a 3.5-fold increase per hour or about 2.3

times the rate at 30°C whereas the rate at which phage concentration increases drops to a 25 fold increase per hour

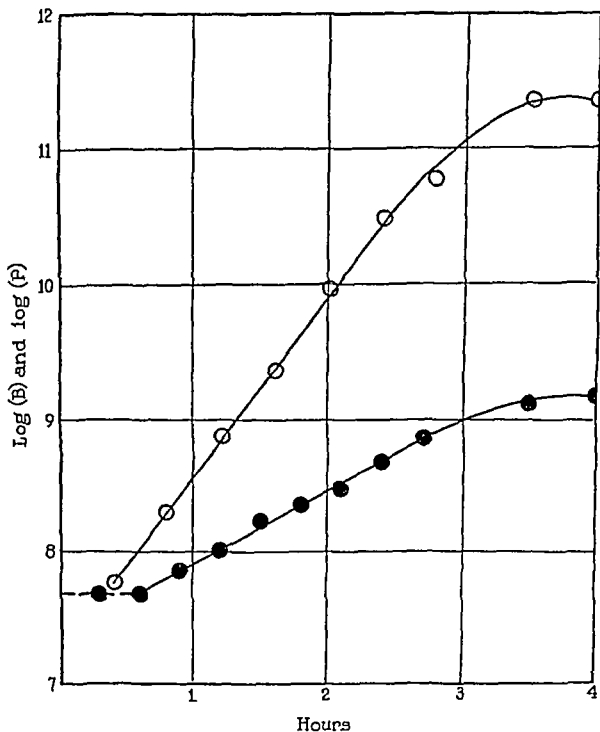


FIG 3 Temperature 40°C

At 45°C the phase of logarithmic bacterial growth drops to a 3 fold increase per hour while phage concentration instead of increasing definitely decreases

## 2 Effect of pH on the Phage-Bacterium Reaction

Figs 5-9 illustrate graphically the influence of H-ion concentrations from 6 to 8.5 on phage production and bacterial growth at 36°C. It is evident that with increasing concentrations of H-ions the lag phase of bacterial growth is prolonged, although the rate of logarithmic

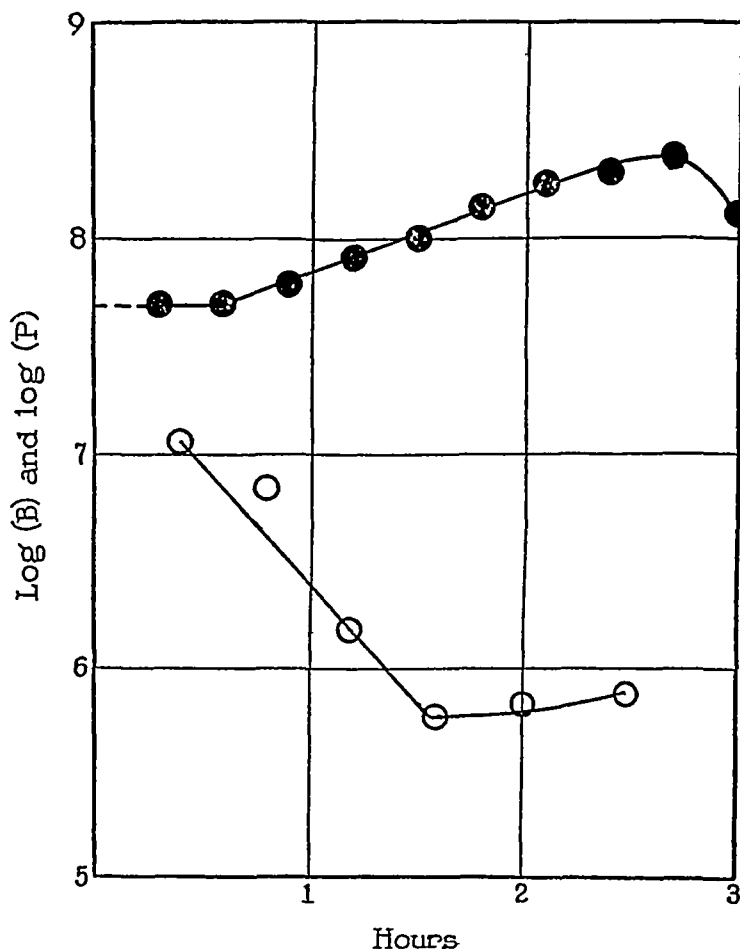


FIG 4 Temperature 45°C

increase when once begun is not appreciably altered. Likewise, the rate of phage production is not changed at lower pH's. While a change in pH from 7 to 6 results in increasing the lag phase of bacterial growth from 0.25 hour to 1.25 hours, there is no corresponding increase in the lag phase of phage production. As the H-ion concentration is

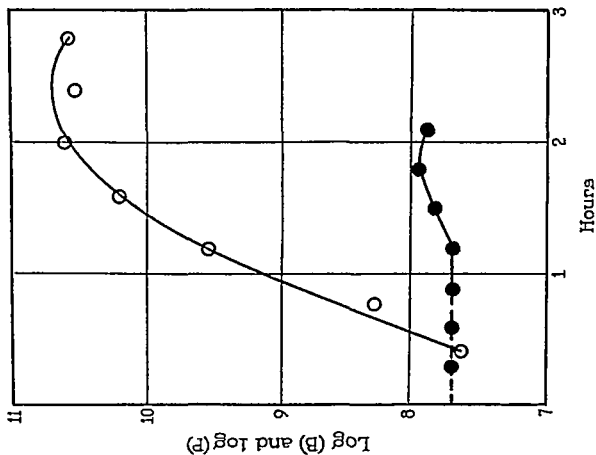


FIG 5 pH 6

FIGS 5-9 Effect of pH on the phage bacterium reaction. Phage production and bacterial growth at 36°C and at various pH values are plotted against time. O = phage activity units/ml. ● = bacteria per ml.

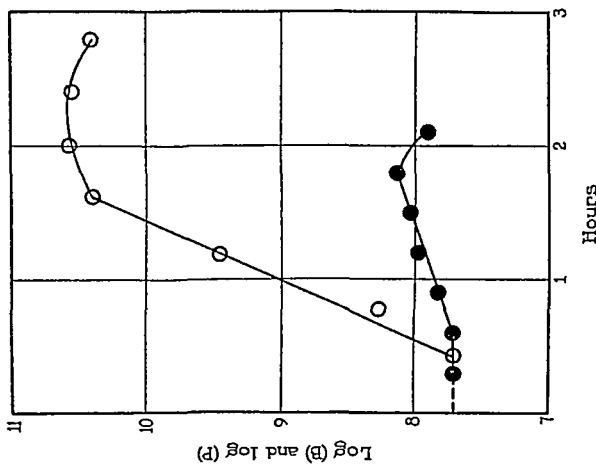
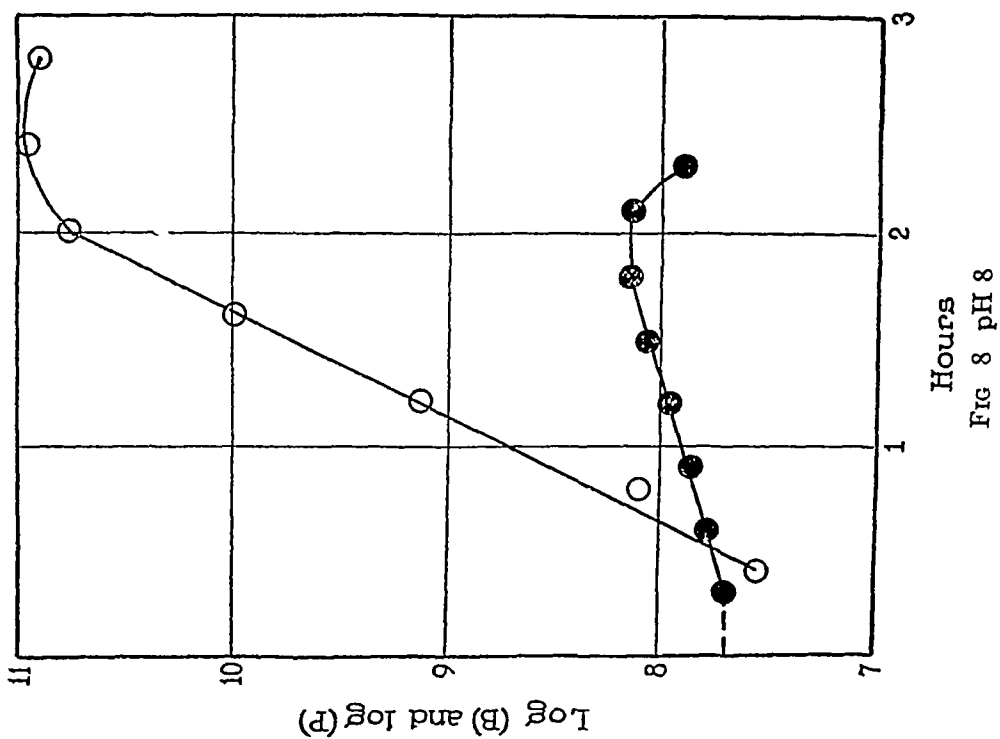
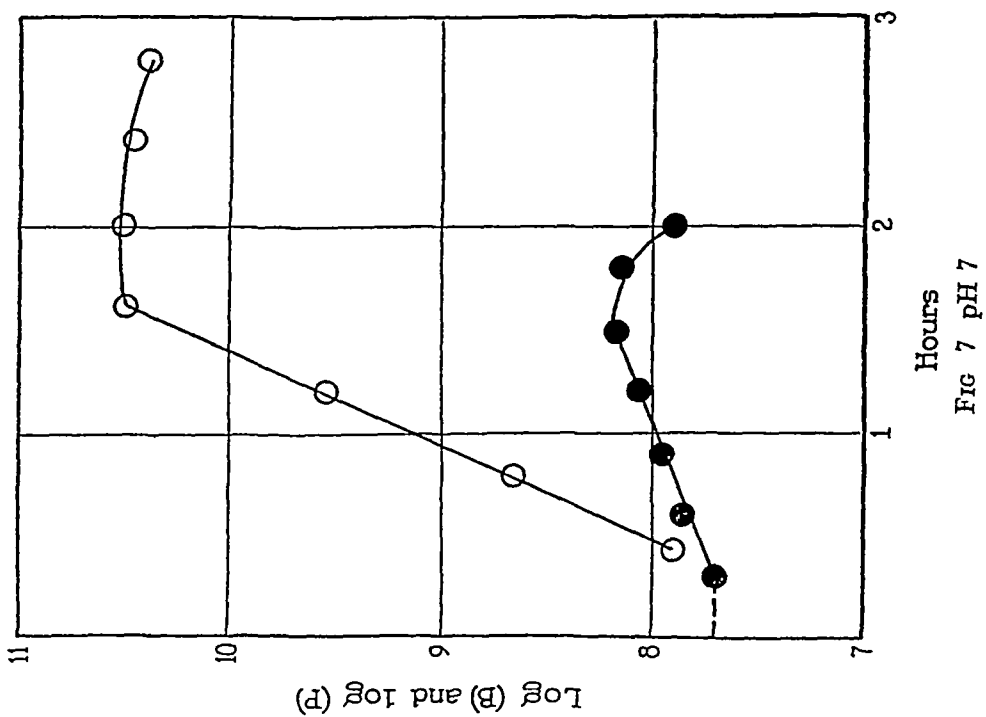


FIG 6 pH 6.5

FIGS 5-9 Effect of pH on the phage bacterium reaction. Phage production and bacterial growth at 36°C and at various pH values are plotted against time. O = phage activity units/ml. ● = bacteria per ml.



decreased the only noticeable change is a slight drop in the rate of bacterial reproduction occurring at pH 8.5, it is not accompanied by any alteration in the rate of phage production

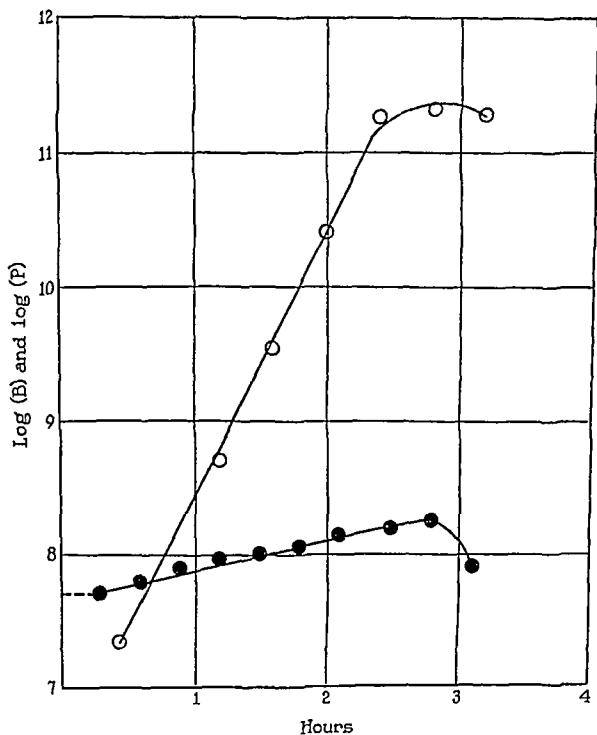


FIG 9 pH 8.5

In the experiments performed at pH 6 and 36°C [phage] increased over 10 fold before there was any detectable increase in [bacteria]

To see if this apparent dissociation between the processes of bacterial reproduction and phage formation could be made more evident several identical experiments were carried out at pH 6 with the temperature adjusted to 28°C instead of 36°C. In Fig 10 the general results are

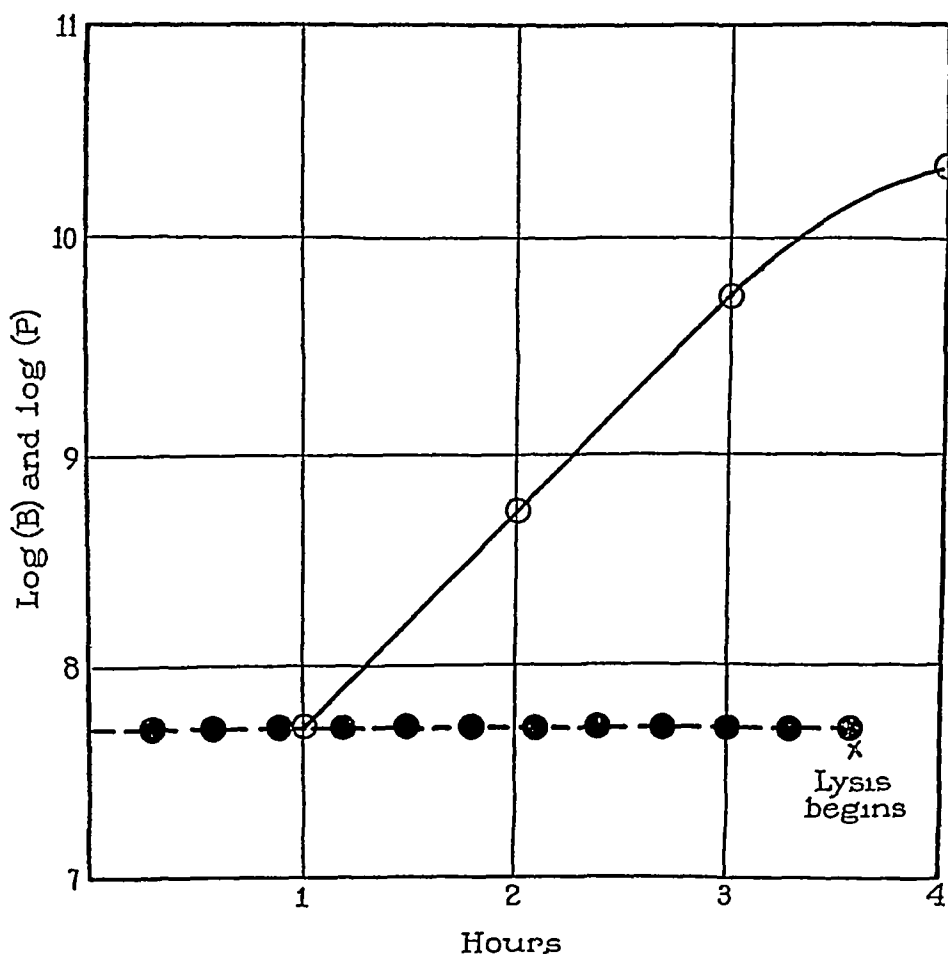


FIG 10 Phage production in the absence of bacterial growth [Phage] shows a 10-fold increase per hour while the bacteria show no growth at all ○ = phage activity units/ml ● = bacteria per ml

plotted and it is clear that under these conditions no observable increase in [bacteria] occurs while [phage] rises over 100-fold

#### DISCUSSION

From the data of the experiments performed at different temperatures, it follows that the processes of bacterial reproduction and phage

formation are not related in the simple way previously reported. The equation used by Krueger and Northrop (1) to express phage production in terms of bacterial growth stated that

$$P/P_0 = (B/B_0)$$

Where  $B$  = bacterial concentration at any time,  $B_0$  = initial bacterial concentration,  $P$  = phage concentration at any time, and  $P_0$  = initial phage concentration. This equation fits the data for which it was derived quite satisfactorily but in the experiments here reported it does not apply. That is, while the rate of bacterial growth shows a regular increment with increasing temperature up to  $40^\circ\text{C}$ , phage production exhibits a marked rise in rate as the temperature is raised from  $30^\circ\text{C}$  to  $35^\circ\text{C}$  but at  $40^\circ\text{C}$  the rate of phage formation drops markedly. At this last temperature the time of lysis is delayed. With a further rise in temperature to  $45^\circ\text{C}$  the logarithmic phase of bacterial growth shows a 3 fold increase in population per hour, a somewhat lower rate than that observed at  $40^\circ\text{C}$ . The concentration of phage in the mixture instead of increasing definitely diminishes. This may be ascribed to one of two mechanisms. First it is evident that  $45^\circ\text{C}$  is beyond the optimum temperature for growth of this particular staphylococcus and it is likely that a good many dead cells are accumulating in the suspension. Dead bacteria adsorb phage irreversibly in quite large amounts (6) and the drop in phage may well be due to such adsorption. Second the loss of phage may be attributed to direct heat inactivation. From the previously reported data of Krueger (7) it is known that this phage does not become inactivated at any such rate until higher temperatures are employed. The first mechanism, namely, removal of phage by adsorption to dead cells seems the most probable one, for after a pronounced drop in [phage] there follows a phase of slow increase in [phage] as might be expected after the available dead cell surfaces had become saturated.

The main feature of the temperature experiments is the fact that the rate of phage production shows an optimum in the neighborhood of  $35^\circ\text{C}$  and the bacterial growth optimum is about  $40^\circ\text{C}$ . While the rate of bacterial growth rises as the temperature is raised from  $35^\circ\text{C}$  to  $40^\circ\text{C}$  the rate of phage formation exhibits a pronounced drop.

In the experiments on the effects of H ion concentration on the



phage-bacterium reaction the most marked differences are observed in the acid range. As  $[H-ion]$  increases the lag phase of bacterial growth is increased, although the slope of the phase of logarithmic growth is not appreciably altered when once begun. At pH 6.0 bacterial growth occurs for only 0.6 hour as compared with the logarithmic growth phase of 1.2 hours at pH 7. Likewise, only one-half the total number of cells is produced at pH 6 as at pH 7. Nevertheless, there is no corresponding increase in the lag phase of phage production nor is the rate of phage formation altered by lower pH's. The total amount of phage formed at pH 6.0 and pH 6.5 is identical with that produced at pH 7.0.

With increasing alkalinity there appears to be no pronounced deviation from the normal curves of bacterial growth and phage formation. At pH 8.5 lysis is delayed almost an hour, and the lytic threshold is in the neighborhood of 1000 activity units per bacterium instead of 100-140 as is usually the case.

The pH experiments indicate that phage formation can occur at the normal rate in the absence of bacterial growth (see Fig. 5). In another experiment at pH 6 using a temperature of 28°C instead of 36°C we found that phage formation goes on at the rate of a 10-fold increase per hour, while there is no bacterial growth at all during the entire experimental period.

From the above considerations it would appear that the significance previously accorded bacterial growth as the essential conditioning factor for phage production may be questioned. At pH 7.4 and 36°C the equation expressing phage production in terms of the rate of bacterial growth fits the observed experimental data very well (1). The reason for this good agreement is probably to be found in the fact that both the phage-forming mechanism and the mechanism of bacterial growth happen to follow the course of autocatalytic reactions. The apparent relationship is further emphasized by the cessation of phage formation when bacterial growth is stopped abruptly by altering environmental conditions, *e.g.* shifting to very low temperatures as was done by Krueger and Northrop (1). The dependence of phage production on bacterial growth is, however, more apparent than real, for the two phenomena can be dissociated. When bacterial reproduction is inhibited by the use of broth adjusted to

pH 6 and 28°C phage production nevertheless proceeds at a rapid rate (Fig. 10). The selection of bacterial growth data for use in deriving the equation for phage production was then merely fortuitous and basically without significance. In place of the expression for bacterial growth there should be substituted the terms for some other reaction which proceeds logarithmically with time but whose nature is at present unknown. This reaction may well consist of the production of a phage precursor which is promptly converted into phage by phage itself. Numerous unpublished observations made in this laboratory over the past 3 years have shown that 100 per cent increases in phage titre can be obtained by adding certain cell free ultrafiltrates of bacterial preparations of phage.

There is good reason to believe that phage is a protein with the properties of an enzyme (7-11). We feel that the experiments reported in the present paper support this concept, and suggest that the mechanism of phage production can be studied like any other cellular mechanism of enzyme formation under conditions which set it apart from the complexities of cellular growth.

#### SUMMARY AND CONCLUSIONS

1. The effects of temperature and H<sup>+</sup> ion concentration on the reaction between antistaphylococcus phage and a susceptible staphylococcus have been studied.

2. The temperature optimum for phage production is in the neighborhood of 35°C and that for bacterial growth is approximately 40°C.

3. With increasing H<sup>+</sup> ion concentrations there occur (a) an increase in the lag phase of bacterial growth without any corresponding increase in the lag phase of phage production, (b) a diminution in the total bacterial population accumulating in the medium without any corresponding drop in the total amount of phage formed.

4. With increasing alkalinity there is no pronounced change in the curves of bacterial growth and phage formation. At pH 8.5 the lytic threshold is increased to about 1000 phage units per bacterium instead of 100-140 as is usually the case and the time of lysis is delayed.

5. By adjusting the medium to pH 6 and 28°C bacterial growth can be completely inhibited while phage production continues at a rapid rate.

6 Apparently, the previously stressed importance of bacterial growth as the prime conditioning factor for phage formation does not hold, for under certain experimental conditions the two mechanisms can be dissociated

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# THE INDUCTION PERIOD IN PHOTOSYNTHESIS

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## I

### INTRODUCTION

When a plant is illuminated, its rate of photosynthesis is at first low and gradually increases until it becomes constant. This induction period was first observed by Osterhout and Haas (1918) for *Ulva* and independently confirmed by Warburg (1920) with *Chlorella*. It has since been found by Van der Paauw (1932) for *Hormidium*, by Briggs (1933) for *Mnium*, and by Emerson and Green for *Gigartina* (1934). It is even demonstrable in Willstätter and Stoll's (1918) measurements with *Helianthus*, *Sambucus*, and *Acer*. Though present in such a variety of plants, the induction period varies considerably, being 2 minutes in *Chlorella* and *Hormidium*, 20 minutes in *Gigartina*, 50 minutes in *Mnium*, and even longer in *Ulva*. Van der Paauw found its duration to vary with temperature.

The mere existence of the induction period demonstrates that the light process in photosynthesis must precede the dark or Blackman process (Warburg, 1920, Baly, 1934). We have therefore undertaken a quantitative description of it under various conditions in the hope that it will give further information about the processes involved in photosynthesis.

## II

### EXPERIMENTAL

*1 Procedure*—The fresh water plant *Cabomba caroliniana* was used with the same methods for control and measurement of photosynthesis as in a previous research (Smith 1937).

The tissue was placed in carbonate bicarbonate mixtures in a Warburg vessel and after a short time in the dark its respiration determined for 30 minutes. As

Warburg has noted, it is not possible to get accurate measurements by periodic observation of the manometer during continuous illumination, there is always a definite lag in the liberation of oxygen from the solution. The plant was therefore exposed for 1 minute to the light, and after 5 or 10 minutes in the dark the reading of the manometer was taken. The procedure was then repeated for successively longer light exposures, until a complete set of data was obtained on

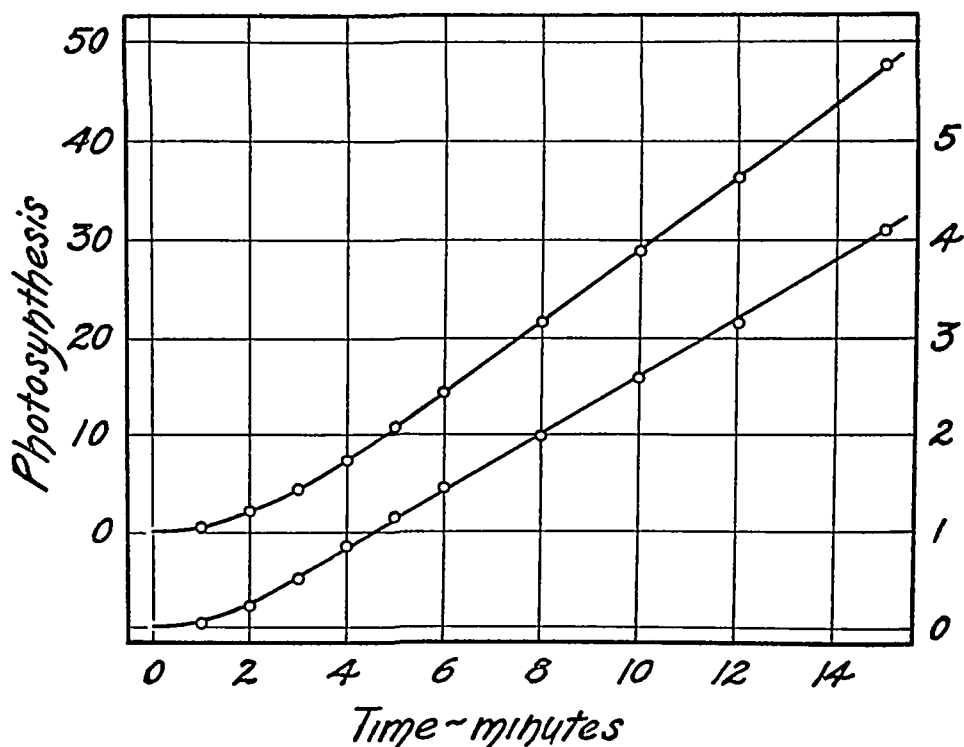


FIG 1 Photosynthesis as a function of time of illumination for *Cabomba*. The upper curve is for an intensity of 282,000 meter candles, the lower one for 1,740 meter candles. An induction period is present at both illuminations. The data are averages, each curve representing five similar runs, they are given in Table I.

the particular tissue. Several readings were usually made for the short exposures. Where the total amount of photosynthesis was small, three or four fronds of about 300 mg wet weight were used, otherwise a single frond of about 100 mg was adequate. A complete run was always made with each tissue, and repeated four times with different fronds so that each point represents the average of at least five individual readings, while the points for short exposures include several more. The average data are in no way different from the single runs.

2 *Results*—Warburg was unable to find an induction period with *Chlorella* at low intensities. This is not the case for *Cabomba*. Fig 1 presents the data for a high and a low light intensity, both show a well marked induction period. These data are in Table I together with those for an intermediate intensity and for two lower CO<sub>2</sub> concentrations at high intensity. For *Cabomba*, photosynthesis balances respiration near 300 meter candles, reliable measurements for short exposures are thus not possible much below 1500 meter candles. Above this, the induction period is demonstrable over a range of

TABLE I  
*Photosynthesis As a Function of Time of Illumination*

Each set of data is the average of 5 similar experiments. Photosynthesis from the beginning of the illumination given as cubic millimeters of oxygen evolved per 100 mg wet weight of tissue, corrected for respiration. Intensities are in meter candles and CO<sub>2</sub> concentrations in moles per liter.

Time <i>min</i>	Photosynthesis				
	[CO <sub>2</sub> ] = $2.90 \times 10^{-4}$ <i>I</i> = 1 740	[CO <sub>2</sub> ] = $2.90 \times 10^{-4}$ <i>I</i> = 11 800	[CO <sub>2</sub> ] = $2.90 \times 10^{-4}$ <i>I</i> = 282 000	[CO <sub>2</sub> ] = $7.87 \times 10^{-4}$ <i>I</i> = 282 000	[CO <sub>2</sub> ] = $2.05 \times 10^{-3}$ <i>I</i> = 282 000
1	0 05	0 29	0 49	0 27	0 15
2	0 23	1 12	2 06	1 22	0 51
3	0 51	2 41	4 36	2 69	1 04
4	0 85	3 99	7 34	4 40	1 59
5	1 15	5 86	10 79	6 09	2 27
6	1 46	7 72	14 30	7 81	2 95
8	1 99	11 52	21 74	11 17	4 16
10	2 60	15 33	28 94	14 47	5 38
12	3 16	19 09	36 28	17 85	6 71
15	4 09	24 74	47 56	23 02	8 48

intensities of about 1 to 160 (from 1,740 to 282,000 meter candles) which at this high CO<sub>2</sub> concentration is about 85 per cent of the total photosynthesis range. Approximately the same range is covered by the three experiments at different CO<sub>2</sub> concentrations at high light intensity (Smith, 1937).

The data of the induction period are well described by the equation

$$\log \frac{p_m^{\frac{1}{2}} + p^{\frac{1}{2}}}{p_m^{\frac{1}{2}} - p^{\frac{1}{2}}} = Kt \quad (1)$$

where  $p$  is the photosynthesis rate at any time ( $t$ ), and  $p_m$  is the maximum rate. When plotted on a double logarithmic scale, the shape

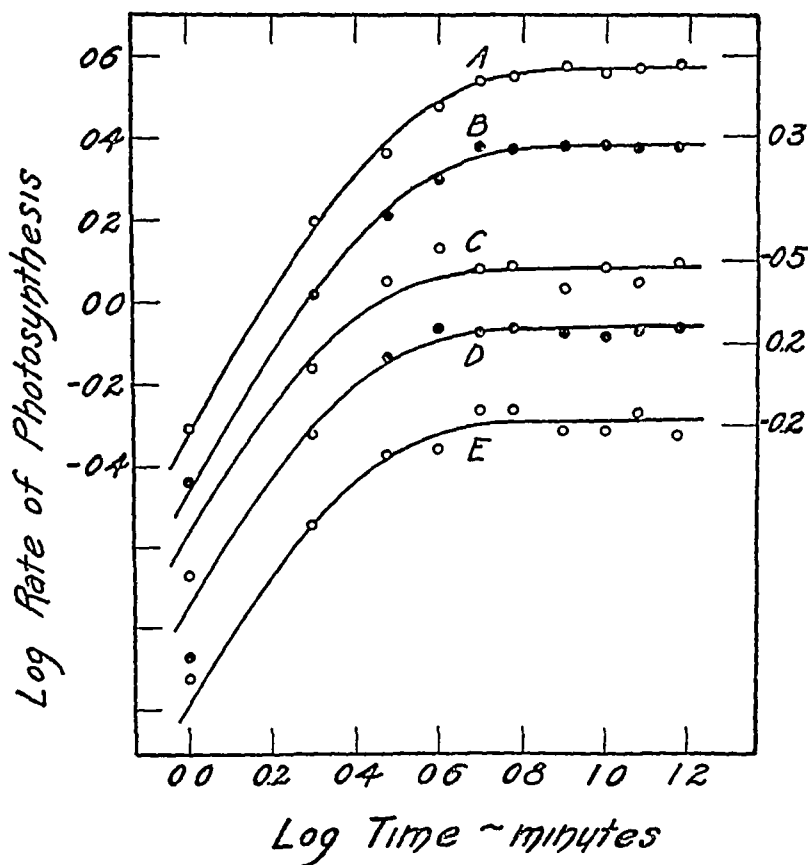


FIG. 2 Rate of photosynthesis as a function of time for different intensities and  $\text{CO}_2$  concentrations for *Cabomba*. The same curve is drawn through all the data and is from equation (1). Photosynthesis is in cubic millimeters of oxygen per minute with the scale correct only for curve A, the others have been displaced by different amounts, with the correct positions indicated on the right side of the figure. The light intensities ( $I$ ) in meter candles, and the  $\text{CO}_2$  concentrations in moles per liter are as follows: (A)  $I = 282,000$ ,  $[\text{CO}_2] = 2.90 \times 10^{-4}$ ; (B)  $I = 11,800$ ,  $[\text{CO}_2] = 2.90 \times 10^{-4}$ ; (C)  $I = 1,740$ ,  $[\text{CO}_2] = 2.90 \times 10^{-4}$ ; (D)  $I = 282,000$ ,  $[\text{CO}_2] = 7.87 \times 10^{-5}$ ; (E)  $I = 282,000$ ,  $[\text{CO}_2] = 2.05 \times 10^{-5}$ . These data are taken from Table I.

of the curve of this equation is independent of the constants  $p_m$  and  $K$ . In Fig. 2 this curve is drawn through all the measurements for

*Cabomba* given in Table I, so that a change in light intensity or  $\text{CO}_2$  concentration affects only the position of the curve but not its character. Table II gives the photosynthesis rates as oxygen produced per minute, together with the values calculated from equation (1).

Equation (1) also describes with good precision the data of Warburg and of Briggs drawn in Fig. 3. Thus the measurements obtained on three plants, *Cabomba*, *Chlorella*, and *Monium*, each representative of different phyla, are shown to be similar. Since the effect of light

TABLE II  
*Rate of Photosynthesis and Time*

Observed values from Table I expressed as oxygen produced per minute. Calculated values are from equation (1) with the constants obtained by graphical fit.

Time min	$[\text{CO}_2] = 2.90 \times 10^{-4}$ $I = 1,740$ $p_m = 0.303 \text{ } K = 0.452$		$[\text{CO}_2] = 2.90 \times 10^{-4}$ $I = 11,800$ $p_m = 1.90 \text{ } K = 0.345$		$[\text{CO}_2] = 2.90 \times 10^{-4}$ $I = 282,000$ $p_m = 3.68 \text{ } K = 0.325$		$[\text{CO}_2] = 7.87 \times 10^{-4}$ $I = 282,000$ $p_m = 1.73 \text{ } K = 0.423$		$[\text{CO}_2] = 2.05 \times 10^{-4}$ $I = 282,000$ $p_m = 0.646 \text{ } K = 0.419$	
	$p_{be}$	$p_{calc}$	$p_{be}$	$p_{calc}$	$p_{be}$	$p_{lc}$	$p_{be}$	$p_{l}$	$p_{be}$	$p_{calc}$
1	0.05	0.07	0.29	0.27	0.49	0.47	0.27	0.36	0.15	0.13
2	0.17	0.18	0.83	0.83	1.57	1.48	0.95	0.99	0.36	0.36
3	0.28	0.25	1.29	1.31	2.30	2.40	1.47	1.40	0.53	0.52
4	0.34	0.28	1.58	1.61	2.98	3.01	1.71	1.60	0.55	0.59
5	0.30	0.30	1.87	1.76	3.45	3.35	1.69	1.68	0.68	0.63
6	0.31	0.30	1.86	1.84	3.51	3.52	1.72	1.71	0.68	0.64
8	0.27	0.30	1.90	1.89	3.72	3.64	1.68	1.73	0.61	0.65
10	0.31	0.30	1.91	1.90	3.60	3.67	1.65	1.73	0.61	0.65
12	0.28	0.30	1.88	1.90	3.67	3.68	1.69	1.73	0.67	0.65
15	0.31	0.30	1.88	1.90	3.76	3.68	1.72	1.73	0.59	0.65

intensity and  $\text{CO}_2$  concentration is the same for all plants which have been investigated (Smith, 1936, 1937), the induction period provides an additional aspect of the similarity of the photosynthetic mechanism in different plants.

In Fig. 4 are plotted the data for *Hormidium* for the three temperatures studied by Van der Paauw. Although of lower precision than the others, these measurements are consistent with equation (1). The large shift of the curves on the time axis with an increase in temperature suggests that it is not a photochemical or a diffusion process.



which is rate limiting Both the time shift and the increase in the final maximum involve only a change in the constants which describe the data

Emerson and Green's measurements made with *Gigartina* immersed in sea water are well described by equation (1) However, equation (1) does not fit the data obtained when the plant was immersed in sea

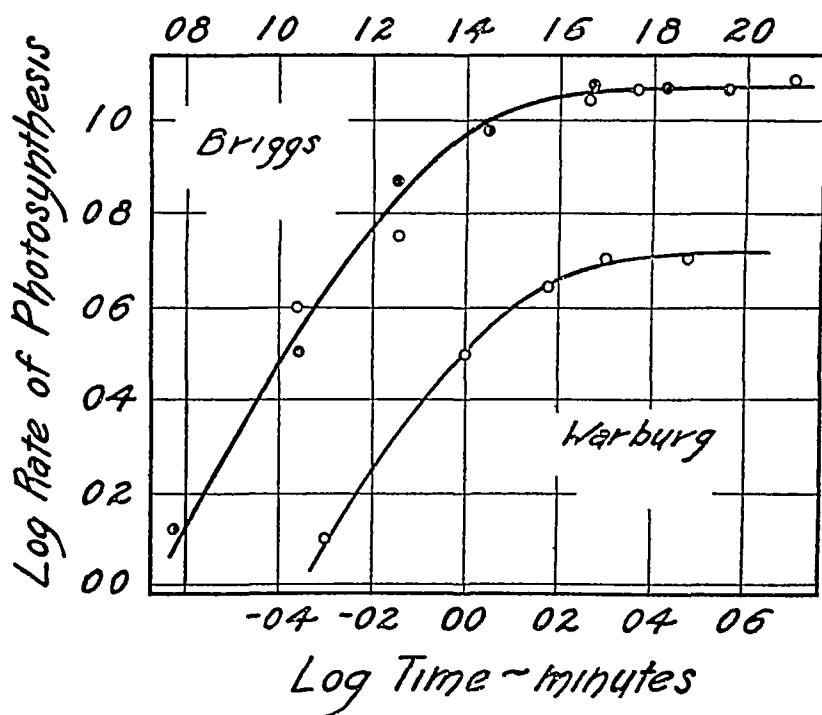


FIG 3 Rate of photosynthesis as a function of time The upper set of data are for two runs, *DI* (open circles) and *EV* (solid circles) on *Mnium* by Briggs (1933) with the correct time scale indicated at the top of the figure The lower set of data are those of Warburg (1920) on *Chlorella* The photosynthesis scale is arbitrary The same curve is drawn through the data for both plants and is from equation (1)

water saturated with 5 per cent carbon dioxide in air The latter data show a longer induction time but reach the same maximum as that attained in sea water The different curves probably represent some special effect, since the observations on other species are similar in spite of different experimental conditions The measurements of Osterhout and Haas who first observed this induction phenomenon are

not sufficiently reliable for comparison with other data, because their data are vitiated by lack of suitable control of the  $\text{CO}_2$  concentration, a variable which produces large changes in photosynthesis rate

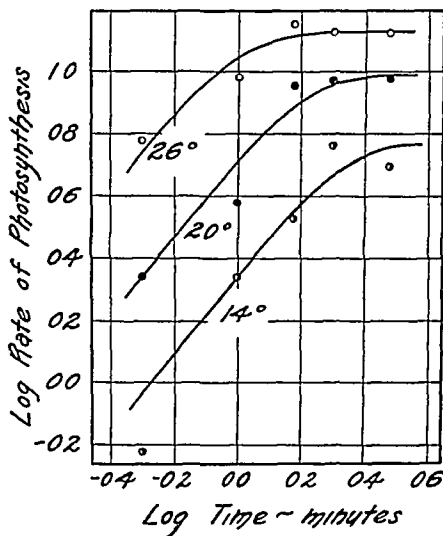


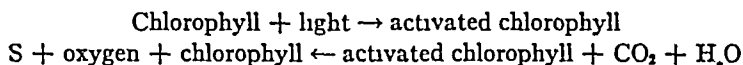
FIG 4 The data of Van der Paauw (1932) on *Hormidium* for the relation between photosynthesis rate and time at three temperatures. Photosynthesis is given as rate per minute in Van der Paauw's units. For the 26 data a value at 2.0 minutes has been interpolated in place of an experimental point that is obviously out of line with the rest of the data. The curve from equation (1) has been drawn through the three sets of data. A change of temperature does not alter the shape but only the position of the curve with respect to the ordinates.

### III

#### Theoretical

Equation (1) which describes the data of the induction period may be derived by considering the rôle of chlorophyll in the cycle of light

and dark reactions <sup>1</sup> Chlorophyll is apparently involved in the photochemical reactions by the absorption of light quanta, and in the Blackman reaction by transfer of energy affecting the reduction of carbon dioxide The cycle may be pictured



This scheme involves no assumptions regarding the intimate nature of the reactions concerned, and represents merely a minimum picture of the changes which take place The position of the substances other than chlorophyll in this scheme will not affect the equations to be derived since these are constant during a study of the induction period, although both the CO<sub>2</sub> (Emerson and Green, 1934) and the water (Pratt, Craig, and Trelease, 1937) are probably involved in the dark stage *S* represents the carbohydrate formed

The existence of the induction period indicates that the light process precedes, therefore it is the dark reaction which determines photosynthesis, and we may write

$$p = f_2(x) \quad (2)$$

where *p* is the rate of photosynthesis, and *x* is the concentration of activated chlorophyll The concentration of activated chlorophyll depends on the difference in rates of light and dark reactions, since activated chlorophyll is formed in the light and used up in the dark Thus

$$dx/dt = f_1(I, \text{unactivated chlorophyll}) - f_2(x) \quad (3)$$

The relation between *x* and *t* is the integral of equation (3)

<sup>1</sup> Gaffron (1935) has suggested that the diminished oxygen production during the induction period is caused in part by the photo-oxidation of metabolites which accumulate while the plant is in the dark Such a photo-oxidation would require a longer induction time at low intensities As Warburg has pointed out, this would be similar to the induction period present in the hydrogen-chlorine reaction, which is distinctly not the case for photosynthesis We therefore assume that the induction period is a real property of the photosynthetic mechanism as such

If for (2) we write

$$p = k_2 x^2 \quad (4)$$

and for the integral of (3)

$$\int dx/dt = \int k_1(a^2 - x^2) - k_2 x^2 \quad (5)$$

we can derive equation (1) Light intensity, water, and carbon dioxide are constant in any experiment, and are included in the velocity constants  $k_1$  and  $k_2$ ,  $a$  is a constant which may be related to the total available chlorophyll

Carrying out the integration required by (5) and the substitution from (4), we obtain

$$\ln \frac{\left[ \frac{k_1^{\frac{1}{2}} k_2^{\frac{1}{2}} a}{(k_1 + k_2)^{\frac{1}{2}}} \right] + p^{\frac{1}{2}}}{\left[ \frac{k_1^{\frac{1}{2}} k_2^{\frac{1}{2}} a}{(k_1 + k_2)^{\frac{1}{2}}} \right] - p^{\frac{1}{2}}} = 2k_1^{\frac{1}{2}} a (k_1 + k_2)^{\frac{1}{2}} t \quad (6)$$

Calling

$$\frac{k_1^{\frac{1}{2}} k_2^{\frac{1}{2}} a}{(k_1 + k_2)^{\frac{1}{2}}} = p_m^{\frac{1}{2}} \quad \text{and} \quad \frac{2k_1^{\frac{1}{2}} a (k_1 + k_2)^{\frac{1}{2}}}{2.303} = K^*$$

we obtain equation (1)

$$\log \frac{p_m^{\frac{1}{2}} + p^{\frac{1}{2}}}{p_m^{\frac{1}{2}} - p^{\frac{1}{2}}} = Kt$$

\* If  $k_1$  and  $k_2$  the constants for the light and dark processes include the light intensity and  $\text{CO}_2$  concentration respectively then an increase in either of these factors indicates an increase in  $p_m$  the stationary state rate, which is actually the case (Table II) However, the  $K$  values which are also expected to increase, seem to show a trend in the opposite direction The values are too uncertain for any definite conclusion to be drawn since each set of measurements was made on different material

Many curves have been derived from equations similar to (4) and (5) but having different exponents These curves are invariant in form when plotted on a double logarithmic scale, and may readily be compared with the data They all show a steeply rising portion, a rapidly curving transition region, and a horizontal section at the

stationary state The slope of the steep portion depends very largely on the exponent in the term of the dark reaction The approximate slopes are 0.5, 1.0, and 1.5 where  $\lambda$  is respectively raised to the powers 0.5 and 1 and 2 The principal effect of the different exponents in the light reaction is to alter the curvature of the transition region

The slope of the steep portion of the curve is 1.5 (Figs. 2, 3, and 4), indicating that the dark reaction is of the second order When the equations are of simple bimolecular form as in

$$\int dv/dt = \int k_1(a-x)^2 - k_2x^2 \quad (7)$$

with

$$p = k_2x^2, \quad (8)$$

the integral obtained, with the substitution required by (8), gives a slope for the steep portion similar to that of the curve derived from the data and of equation (6), but differs markedly in the curvature at the transition region

Where the light and dark processes are first order as in

$$\int dx/dt = \int k_1(a-x) - k_2x \quad (9)$$

with

$$p = k_2x \quad (10)$$

the resultant equation

$$\ln \left[ 1 - \frac{k_1k_2a}{(k_1 + k_2)p} \right] = (k_1 + k_2)t \quad (11)$$

has a slope of 1.0 for the steep portion, and is wholly inadequate for a description of the data In Fig. 5 are drawn for comparison the curves for equations (6) and (11), and the integral of (7) with the substitution required by (8)

That the simple assumptions of light and dark processes are not completely adequate is shown by the fact that the equations which describe the data for the induction period are not in harmony with those that describe the relation between intensity and photosynthesis

at the stationary state The data for intensity and photosynthesis at the stationary state can be described by the equation<sup>2</sup>

$$dx/dt = k_1 I(a^2 - x^2) - k_2 x^2 = 0 \quad (12)$$

with

$$p = k_2 x \quad (13)$$

whereas, for the induction period,  $p$  must be proportional to  $x^2$  in equation (13) This might indicate that the dark reaction which

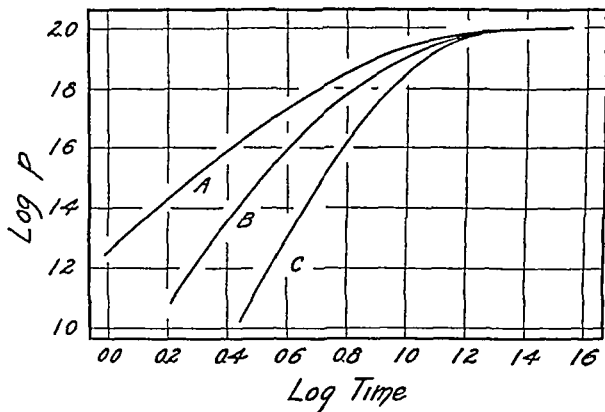


FIG 5 Theoretical curves for the course of photosynthesis rate with time A is from equation (11), B is from the integral of (7) with the substitution of (8) C is from equation (6) The units are arbitrary and the curves have been made to coincide at the stationary state for comparison Drawn on a logarithmic scale, the curves have a shape which is independent of the constants in the equations

limits the rate at the stationary state is not the same reaction which determines the rate of increase of photosynthesis during the induction period The term for the light process ( $a^2 - x^2$ ) is the same in both

In an earlier research (Smith 1937) it was thought that the light reaction might be half-order, which would have made intensity enter as the first power Squaring the stationary state equation brings the intensity and  $\text{CO}_2$  data in line with the evidence supplied by the induction period

cases, with intensity entering as the square, and indicates that the same light reactions are involved in both phenomena

For the first part of the induction period, *i.e.* until the stationary state is approached, the amount of photosynthesis is proportional to the square of the time. This can be related to the results of Emerson and Arnold (1932) who found that the amount of photosynthesis was independent of light intensity when the product of intensity and time was constant. They assumed that this indicated a photochemical process directly proportional to the intensity. With the short light exposures used by these investigators, well within the time of the induction period of *Chlorella*, photosynthesis is proportional to the square of the time, and their results indicate that the product of the squares of intensity and time is constant. This would be in keeping with equation (12) which indicates that intensity enters as the square in the photochemical reactions of photosynthesis.

The author is grateful to Professor Selig Hecht for much friendly advice and criticism.

#### SUMMARY

1 Measurements on the photosynthesis of *Cabomba caroliniana* show an induction period at low and high light intensities and  $\text{CO}_2$  concentrations.

2 The equation which describes the data for *Cabomba* also describes the data obtained by other investigators on different species. The phenomenon is thus shown to be similar in plants representative of three phyla.

3 A derivation of the induction period equation is made from a consideration of the cycle of light and dark processes known to occur in photosynthesis. The equation indicates that light intensity enters as the square, and that the same light reactions are involved as those which affect the stationary state rates. However, a different dark reaction appears to limit photosynthesis during the induction period.

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# THE RELATION BETWEEN VISUAL ACUITY AND ILLUMINATION

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## I

### INTRODUCTION

The ability of the eye to distinguish detail is dependent, among other things, upon the intensity of the illumination falling upon the object. The measure of this ability is termed visual acuity and is expressed as the reciprocal of the angle (in minutes) subtended by the finest detail distinguishable. Uthoff (1886, 1890) first investigated the relation of visual acuity to illumination over an extensive range of intensities, and several years later Koenig (1897) reinvestigated it in so thorough a manner that his data have become classic. These data show that the relationship between visual acuity and the logarithm of the illumination is sigmoid.

Since then numerous studies have demonstrated that at least three experimental conditions not controlled in the earlier work may have a profound effect upon the results. The first of these concerns pupil size, the second, the distance of the test object from the observer, and third, the brightness and extent of the field surrounding the test object.

The physiological properties of the retina are concerned with apparent brightness rather than with the external brightness. Since the relation of the two is influenced by the pupil, Troland (1916) proposed the photon as a unit of retinal brightness, and expressed it as external brightness in millilamberts times  $10/\pi$  times pupil area in square millimeters. The adequacy of this unit to describe apparent brightness has been questioned by Stiles and Crawford (1933) who showed that the effectiveness of the light in producing brightness sensation falls off markedly when it passes through the more peripheral areas

of the lens This has recently been confirmed by Wright and Nelson (1936) In addition, the size of the pupil affects the image-forming properties of the lens and thus the visual acuity of the eye (Cobb, 1914-15) All previous extensive data suffer on this count since the natural pupil was used Even the most recent contribution in this field (Lythgoe, 1932) is not entirely free from this error in intensity scale In his attempt to evaluate the apparent brightness, Lythgoe measured the pupil area of each observer at each intensity and computed the values in photons As pointed out above, such procedure cannot be considered valid any longer in the light of the data of Stiles and Crawford Lythgoe's data, moreover, lack completeness of range Due to his method of measurement in which the test object is exposed 1.6 sec out of every 9.6 sec, it was impossible to obtain readings at low intensities The rod portion is entirely missing Moreover, he was unable to reach the highest intensities on his surround with the result that the upper portions of his curves are doubtful His experiments with an artificial pupil are also open to criticism in that the condition of a uniform and continuous surround did not obtain in them

Another uncontrolled variable is the distance of the test object Aubert and Forster (Aubert, 1865) have shown that in a series of constellations of different sizes, so arranged that they subtend equal geometric angles at the eye, the smaller constellations are resolvable further out in the periphery than the larger ones Recently Freeman (1932), using a simple visual acuity test, found that larger constellations are more efficient than smaller ones These two apparently contradictory results may be reconciled as one phenomenon on the assumption that the focal length of the refractive apparatus of the eye changes with fixation distance Thus, if the nodal point of the lens moves toward the retina with nearer accommodation, the small constellation would give a smaller image on the retina than would the larger The image of Aubert's smaller constellations would fall nearer to the center of the retina than would be accounted for on external geometrics The data of Roelofs and Zeeman (1919) also show an anomalous effect of stimulus distance, the smaller constellation being more efficient for the greater range of their measurements The data on this phenomenon are quite insufficient for a complete

understanding They are enough, however, to show its existence as a source of error

The effect of the surrounding illumination has been recently demonstrated by Lythgoe and Tansley (1929) and Hecht and Smith (1936) on flicker, by Steinhardt (1936) on intensity discrimination, and by Lythgoe (1932) on visual acuity Evidently an extensive surround equal in brightness to that of the test field is necessary in all measurements in which the eye is in a stationary state of adaptation As a corollary, it is equally necessary that the eye be completely adapted to the intensity at which an observation is to be made

To avoid these various uncontrolled variables, an apparatus was designed which eliminates them This apparatus presents a test object that may be varied continuously in size over a range of about 1/100 at a fixed distance of 1 meter from the eye This test object lies in the center of a field that is  $30^\circ$  in extent, and is observed through an artificial pupil of 2 mm diameter The illumination is varied discontinuously by means of filters in steps of approximately 0.3 log unit

## II

### *Apparatus*

Fig 1 shows the optical system of the apparatus *S*, the source, is an incandescent ball of a tungsten arc An image of the source is focussed on the projection lens system, *P.L.*, by the condenser *C* The projection system consists of a Zeiss "Telenegative" of 6 cm focal length rigidly mounted, and a Zeiss 'Tessar' of 15 cm focal length mounted so that it may be moved along its optic axis for a short distance This constitutes a highly corrected projection system of variable focal length Between the condenser and the projection system there is a test object carriage, *T.O.*, which is movable along the optic axis In addition the carriage can be rotated about its optic axis so that the test object may be presented in a number of different meridians An image of this test object is projected on the field lens *FL* after reflection from a front surface plane mirror not shown in the diagram The test object carriage engages a cam and lever which controls the position of the positive member of the projection system This is so arranged that the image of the test object is always in the same plane. Thus, an image of variable size of the test object is formed in the plane of the field lens and serves as the test object when viewed through the pupil *P*

The field lens focusses an image of the projection system and therefore of the source at the artificial pupil *P* which is a circular aperture 2 mm in diameter The field thus far presented to the eye at the pupil is only about 4 in extent

A surround of  $30^\circ$  is furnished by the spherical front surface mirror  $M$ , having a 30 cm radius of curvature and a 15 cm diameter, with a 21 mm circular hole in the center. Another image of the source is focussed at  $S'$  by the system of lenses,  $C'C'$ . The light is then reflected by a right angle prism and diverges to fill the mirror  $M$ .  $M$  in turn focusses an image of  $S'$  at the pupil.

Just within the artificial pupil are the filters,  $F$ , that control the intensity. The first one is a circular glass with 3 sectors of Wratten neutral filters of densities 0.0, 0.3, and 0.6, the second with 5 sectors of densities 0, 1, 2, 3, and 4, the third with 2 sectors of densities 0 and 4. With these it is possible to achieve an intensity range of 8.6 logarithmic units in steps of 0.3 of a unit. In addition, space is available for one of a series of Wratten monochromat filters for work with isolated spectral regions. In most of the measurements to be reported here, this space was occupied by a neutral filter of density 2. All the filters were calibrated for white light by means of a Martens polarization photometer. The maximum

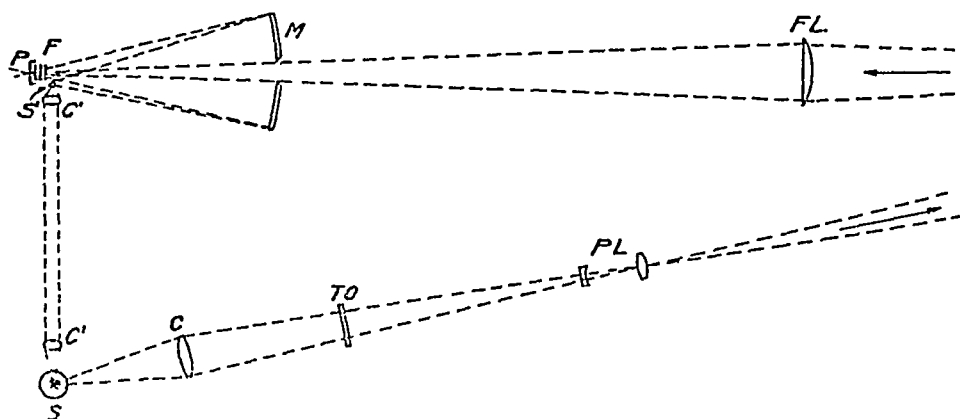


FIG. 1 The diagram of the optical arrangement of the apparatus

intensity obtainable in this apparatus is over 800,000 millilamberts which are equivalent here to over 8,000,000 photons.

All the parts shown in Fig. 1 are mounted on two optical bench bars each 1 meter long. These in turn are bolted to two structural steel bars at the ends, forming a rigid unit. The plane mirror not shown is supported from the narrow end of the main unit by means of a light aluminum and duraluminum frame work. A sheet metal hood to shield the observer from stray light is mounted from the other end. The whole rests on a table top with a three point contact.

The test object carriage is movable on its optical bench bar by means of a coarse screw mounted beneath it. This screw is driven by the observer from a conveniently located knob through a light chain drive. A pawl and ratchet wheel limits this motion to one direction only, namely towards an increase in the test object size. The recorder can easily disengage the test object carriage from its screw drive by releasing a pin to reset the test object. A millimeter scale,

engraved on the optical bench, serves to locate the position of the test object. The total motion of the carriage is 38 cm. This produces a 3 to 1 change in the size of the image at the field lens. A series of 7 test objects, no one larger than twice the previous one, enables us to cover the complete range with adequate overlapping.

The image of a large test object was measured at a series of scale positions. The data were plotted as log visual acuity against the scale readings. A table was then constructed from the smooth curve through these data, giving the log visual acuity for every millimeter of the scale. A logarithm factor for each test object is then added to the table reading to give the exact log visual acuity for each plate at its position on the scale.

Two different types of test objects are available. One of them is a grating of alternate opaque and transparent bars of equal width. This series was engraved on glass disks for us by Max Levy and Company of Philadelphia, Pa. The other is the Landolt's broken circle or C in which the width of the line and the gap is one fifth the total outside diameter of the letter. This series was prepared photographically by a special high resolution method, because the smallest C had to be 0.25 mm in diameter. A negative C of 20 mm diameter was accurately formed of sheet brass. Optical flats were thinly coated with a hot 15 per cent gelatin solution and dried. The gelatin was then sensitized with potassium dichromate and the plate exposed in a camera through the glass so that the insoluble portion would be adjacent to the plate. After washing the unexposed gelatin off with hot water, the remaining gelatin was stained with Heidenhain's iron hematoxylin. This stain, perhaps the most commonly used one in cytology and histology, is not a dye but a solid blue black precipitate. These test objects show a perfectly sharp edge at a magnification of 100 diameters. The graininess becomes just visible at 500 times magnification. They were sealed under cover glasses with Canada balsam and measured under a microscope with a filar micrometer. Chromated gelatin is an extremely insensitive photographic material, by using a condensing lens behind the negative C to focus the image of the source on a fully opened  $f/3.5$  photographic objective, the exposures were cut down to reasonable values.

The constancy of the brightness of the test field was experimentally verified. The illumination of the surround was intercepted by means of an opaque shield placed over  $C'$ , and the light from the test field only was permitted to fall on a Weston photronic cell connected to a sensitive galvanometer. The test object carriage with the smallest C plate was then moved over its entire range. No change in the galvanometer deflection was observable even though a change of  $1/4$  of 1 per cent could be easily detected with this set up.

With the grating test object, an extreme intensity difference of about 20 per cent was observed in different combinations of distance and grating size. This is caused by the fact that the diffraction pattern of the source image falling on the projection lens system is varied in extent by the size and distance of the grating. By inserting two ground glasses between the source and the con-

densers and using these as secondary sources of greater area, most of the variation in intensity was eliminated, the residual change being less than 2 per cent. As a result the maximum intensity obtainable was somewhat lower. In addition, the intensity was cut in half by the use of the grating, since a field covered by an unresolvable grating appears uniformly illuminated with half the intensity. This necessitated the removal of the neutral filter of density 2 described above when measurements with the grating were made.

### III

#### *Procedure*

Measurements were begun at the lowest intensities after the observer was dark adapted for about 20 minutes. The observer kept his right eye as near to the artificial pupil as possible and steadied his head by means of a chin rest. After complete adaptation to the intensity of the field, he brought the test object carriage nearer until resolution of the letter was possible. The test object was then reset by the recorder, and the determination repeated. In most of the measurements with the C, two determinations agreed sufficiently to be considered adequate. For reasons to be presented later, four determinations were made at each intensity with the grating. The intensity was then increased by suitably altering the filters and the process repeated. By this means a complete run was obtained at one sitting of less than 3 hours duration.

The data to be reported here were obtained with two experienced observers, both emmetropes. With the C as test object, A. M. C. was able to cover the entire range well within 3 hours and without undue fatigue. E. L. S. was not so adept, and his measurements began at that illumination at which the cones first control visual acuity.

Measurements with the grating as test object are much more difficult and take more time to make. The unresolved grating presents a uniformly illuminated field offering no point of fixation, with the result that the stripes appear rather suddenly and without warning. An attempt to supply a fixation point by pasting a small circular black paper dot on the field lens was only partially successful in overcoming this difficulty, since the resolution does not take place at the fixation point. This is unlike the C which can be fixated and resolved at the point of fixation.

Another difficulty is the pronounced retinal astigmatism found in every eye thus far examined. It is possible to rotate a just resolved test field so slowly that the observer can follow the direction of rotation, and yet have the field become unresolvable at certain angles. This is probably caused by the necessity of having a fairly large number of retinal elements functioning to resolve a grating as compared with the number required to resolve the opening in the C. On the basis of a distribution of threshold for the various retinal elements as suggested by Hecht (1928), it is probable that the functional elements might be more numerous or denser along one axis than along another. This explanation is borne out by two observations. One is that this astigmatism is more pronounced at the lower intensities where the curve of log visual acuity *versus* log  $I$  is steep, and practically disappears at the higher values where the curve levels off. The other one is the fact that the more easily resolved angle is not constant throughout the range at one sitting nor is it the same at each point at different sittings. Thus, for example, in one run for E L S on June 10, 1936, at a log  $I$  value of  $-1.057$  photons, the values for the log visual acuity were  $-0.529$  for the angle  $45^\circ$  to the right, and  $-0.725$  for  $45^\circ$  to the left, a difference of about  $0.2$  logarithmic unit. At about  $0.7$  logarithmic unit higher in intensity the values were  $-0.173$  for  $45^\circ$  to the right and  $-0.058$  for  $45^\circ$  to the left, a difference about half as large and in the opposite direction. Finally at a log  $I$  value of  $0.118$ , no astigmatism could be detected.

For these reasons it was found necessary to take a reading at each of the four positions of the grating at each intensity. Each value was usually taken only once. This is equivalent to taking only one reading at each intensity with the C. Hence, these data are not so smooth as those taken with the C. Eight readings at each intensity were of course indicated, but the desire to cover a large range at one sitting precluded that. Even so the range that could be covered comfortably was greatly shortened. The measurements, however, were so arranged that one observer, A M C, began his measurements at the lowest intensities and continued as far as possible, while the other, E L S, started at the beginning of the cone range and carried the curve to completion.



## IV

*Results with C as Test Object*

The data are given in Table I. Each datum of log visual acuity is the average of the determinations made in three different complete

TABLE I  
*Visual Acuity and Illumination*

C test object				Grating test object		
Log I in photons	Log visual acuity			Log I in photons	Log visual acuity	
	No special fixation	Central fixation at all intensities above 0.1 photon			A M C	E L S
	A M C	A M C	E L S			
I	II	III	IV	V	VI	VII
-2 433	-1 358	-1 468		-2 804	-1 157	
-2 146	-1 230	-1 283		-2 363	-0 961	
-1 862	-1 121	-1 157		-2 076	-0 899	
-1 413	-1 021	-0 981		-1 792	-0 835	
-1 127	-0 932	-0 896		-1 343	-0 691	-0 698
-0 843	-0 836	-1 004	-1 007	-1 057	-0 531	-0 561
-0 441	-0 651	-0 725	-0 724	-0 773	-0 438	-0 338
-0 154	-0 450	-0 515	-0 518	-0 371	-0 206	-0 174
0 130	-0 260	-0 332	-0 335	-0 084	-0 099	-0 087
0 525	-0 141	-0 132	-0 149	0 200	0 015	-0 032
0 812	-0 054	-0 039	-0 055	0 595	0 096	0 030
1 096	0 042	0 059	0 031	0 882	0 099	0 074
1 488	0 162	0 151	0 125	1 166	0 142	0 124
1 775	0 214	0 212	0 186	1 558	0 158	0 144
2 059	0 271	0 249	0 234	1 845	0 179	0 157
2 507	0 300	0 292	0 270	2 129	0 195	0 192
2 794	0 327	0 315	0 268	2 577		0 208
3 078	0 347	0 343	0 294	2 864		0 207
3 480	0 363	0 367	0 313	3 148		0 231
3 767	0 384	0 371	0 324	3 550		0 238
4 051	0 396	0 390	0 345			
4 446	0 385	0 397	0 340			
4 733	0 382	0 399	0 326			
5 017	0 389	0 415	0 345			

runs. They are given in the form of log *I* and log visual acuity, the same form in which they are recorded from the readings of the apparatus.

The data of column II were obtained in the usual way without

special fixation, so that the most sensitive portion of the eye at each intensity was used. The subjective observations are that the first five values are mediated by the periphery of the eye and represent the function of the rods. Each succeeding value is determined by a region of the retinal periphery nearer the center of the eye than the preceding one. The next four values are determined by cones that are not centrally located. As with the rods, each succeeding one of these

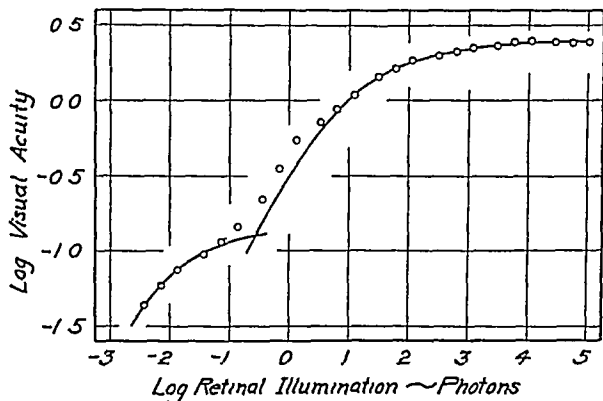


FIG 2 The data of A M C, Table I, column II, showing the relation between log visual acuity with the C and  $\log I$  when the most sensitive portion of the retina is used. The first five points represent the function of the rods, the next four that of the para foveal cones and the remainder that of the central cones. The curve for the rods is of the equation (3) and that for the central cones equation (2).

is determined by a region nearer to the fovea than the preceding one. All the other values are mediated by the fovea of the eye.

To avoid the use of different regions of the retina for each succeeding determination, at least for the cones, the next series of measurements, given in columns III and IV, were made with central fixation as soon as the cones took over the function, namely, at a brightness of about 0.1 photon. This was made possible by the fact that the C test object can be fixated centrally long before resolution of the

opening is possible. This fixation can be maintained readily until central resolution is achieved in spite of the fact that resolution by adjacent regions of the retina may be possible earlier.

The data of column II are plotted in Fig. 2 and those of columns III and IV in Fig. 3, with those of E L S in column IV displaced 0.5 log unit below those of A M C. The logarithm of the visual acuity is plotted against the logarithm of the illumination. Such double

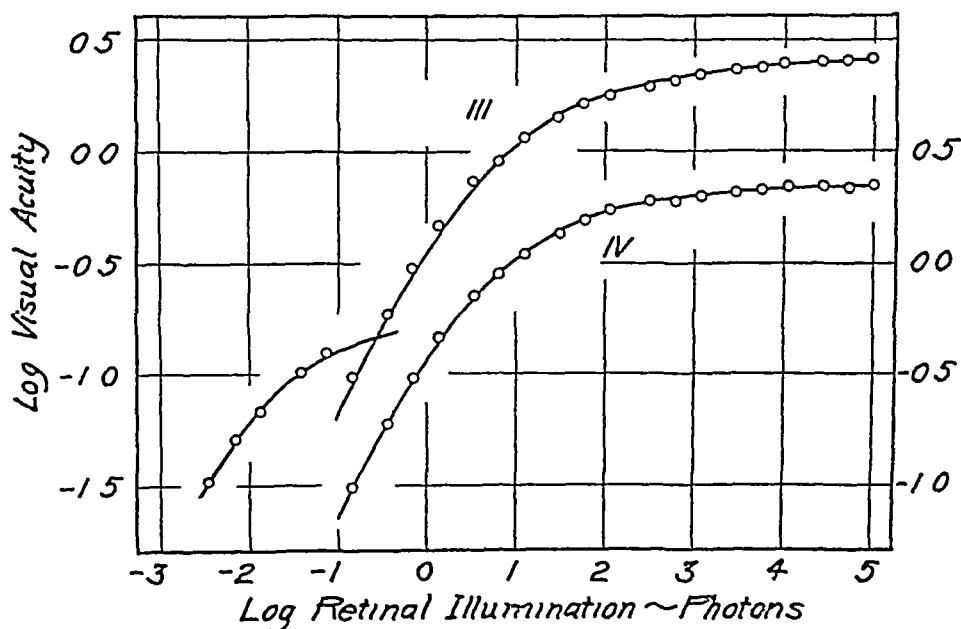


FIG. 3. The data of A M C, Table I, column III, and for E L S, Table I, column IV, showing the relation between log visual acuity with the C and log  $I$  when central fixation is maintained for the entire cone portion of the data. The ordinates for A M C are at the left and those for E L S are at the right. The curve for the rods is of the equation (3), that for the cones, equation (2).

log plots are superior to the single log plots on three counts. One, they serve to bring out more strikingly the discontinuity in the function of rods and cones [see Hecht, 1934 (intensity discrimination), Hecht and Shlaer, 1936, and Hecht and Smith, 1936 (flicker)], and that they do so here may be judged by comparing these double log plots with the single log plots in Fig. 4. Two, this type of plot shows deviations of equal percentage throughout the complete range, and is again illustrated by a comparison with Fig. 4 where the deviations

become larger the higher the visual acuity. Three, in such plots the shape of the curve through the data is independent of the particular units involved. This greatly simplifies the selection of a theoretical curve to describe the data.

The same curve is drawn through all the rod data, and a slightly different one through all the cone data in both these figures. These

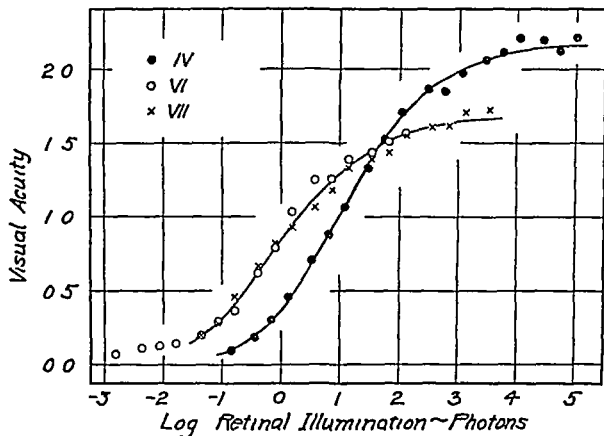


FIG. 4. The single log plot of some of the previously plotted data showing the increased scatter at high visual acuities and the change in slope of the sigmoid curve with change of the maximum visual acuity. The filled circles are the data of E. L. S. Table I, column IV, with the C. The circles are for A. M. C. Table I, column VI, and the crosses are for E. L. S. Table I, column VII, both with the grating. The curves are for the cones only and are of equation (2).

curves are theoretical and will be discussed presently. It may, however, be pointed out here that the subjective observations made during the measurements plotted in Fig. 2 manifest themselves by the fact that the first four cone points, representing the function of a non-homogeneous group of para foveal cones, do not fall on the theoretical curve but lie above it.

The maximum visual acuity for E. L. S. is somewhat lower than that

for A M C In a single log plot as in Fig 4 this manifests itself in a lower slope of the sigmoid curve as well as a lower maximum

The measurements with A M C were on one occasion carried on for about two more logarithmic units in brightness The values of visual acuity neither rose nor fell throughout this change of 100 times in the intensity This is taken to indicate both that the maximum intensity here recorded is adequate to achieve the maximum visual

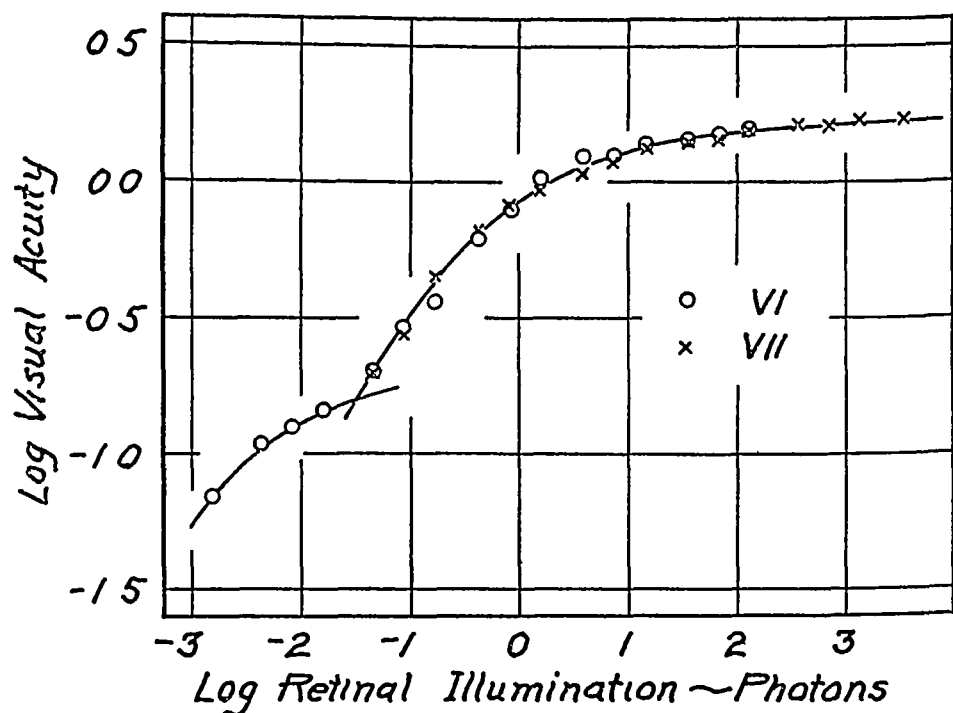


FIG 5 The data of A M C, Table I, column VI, circles, and of E L S, Table I, column VII, crosses, showing the relation between log visual acuity with the grating and log  $I$  The curve for the rods is of equation (3) and that for the cones, equation (2)

acuity of the eye as well as that the surround is adequate in area and in brightness Measurements on various functions of vision (Lythgoe (1932) on visual acuity, Hecht and Smith (1936) on flicker, Hecht (1935) and Steinhardt (1936) on intensity discrimination) have shown that a surround inadequate in either area or intensity or both is invariably manifest by a decrease in sensitivity at the highest intensities

## V

*Results with the Grating as Test Object*

The visual acuity data with a grating as the test object are presented in the last three columns of Table I and plotted in Fig 5. The grating was chosen as a second test object primarily to elucidate the problem of area *versus* distance in detail perception. Should this property be measured by a distance, such as visual acuity, or by an area? For a grating, the area involved is directly proportional to the distance between bars because the length remains unchanged, while for the C the area is proportional to the square of the distance. If area is the basis for detail perception, then visual acuity should be squared for the C, and multiplied by a constant, corresponding to the effective length of the bar, for the grating in order that they be comparable. This means that in a double log plot of visual acuity against intensity of illumination the curve for the C should be one half the slope of that for the grating. The data plotted in Fig 5 are adequately described by the same two curves that described the data with the C test object, showing that detail discrimination is a function of distance rather than area. This justifies fully the use of visual acuity as here defined, and usually used, as a measure of this property of the eye.

## VI

*Factors Limiting the Resolving Power of the Eye for a Grating*

Another reason for the choice of the grating is that it lends itself to a quantitative evaluation of the limiting factors in the resolving power of the eye. The factors that might be operating here are three in number: (1) the "deterioration" of the retinal image as a result of aberrations of the lens and diffraction at the pupil together with the intensity discrimination of the retina, (2) the diameter of the pupil in its relation to the transmission of the diffraction spectra resulting from the test object, and (3) the resolving power of the retina as a function of the separation between retinal elements.

In the case of the grating it is possible to compute the point by point intensity distribution in the retinal image as a result of chromatic aberration of the dioptric system and diffraction pattern of the pupil. The problem is similar to that of an edge of an extensive dark area

adjacent to an extensive light area due to the fact that the bars are very long compared to their width. Such computations with the C test object are very much more difficult and have never been carried out due to the shortness of all dimensions of the opening. Using the

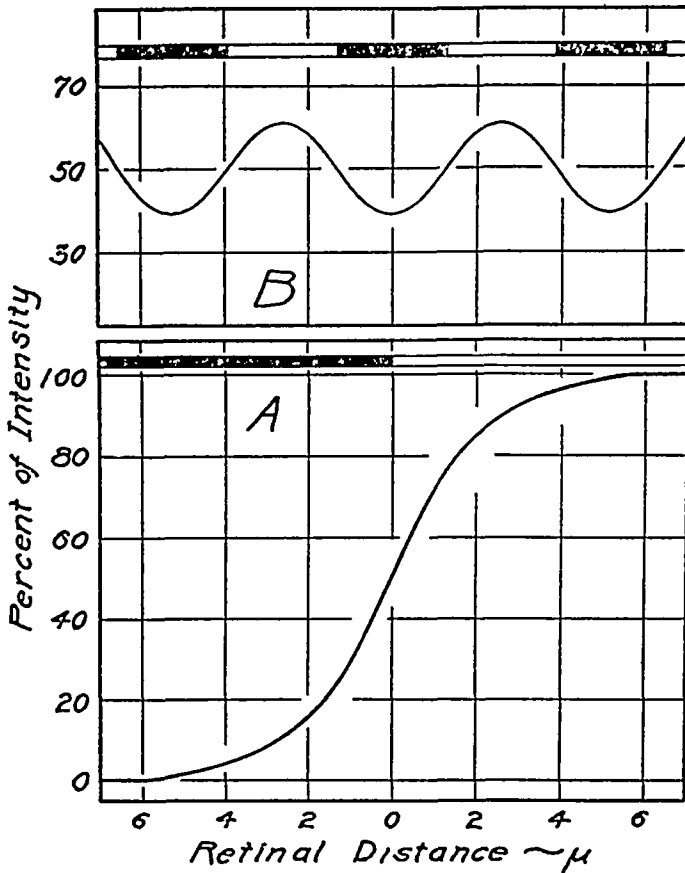


FIG 6 A The distribution of the light in an image of an extensive edge on the retina when chromatic aberration and diffraction are taken into account. Values are from Hartridge (1922)

B The distribution of light in an image of a grating corresponding to a visual acuity of 17 on the retina when chromatic aberration and diffraction are taken into account

curve computed by Hartridge (1922) and reproduced in Fig 6A that gives the distribution of intensity across the retinal image of an edge when chromatic aberration and diffraction are taken into account, the distribution of intensity in the image of the grating corresponding to a

visual acuity of 1.7 was computed. The intensity goes through a sinusoidal distribution around 50 per cent as shown in Fig. 6B. The maxima reach only to 61 per cent, and the minima go down to only 39 per cent. Assuming for the moment that the spacing in the image corresponds to the spacing of adjacent rows of cones in the retina, the average intensities on them is 57 per cent and 43 per cent. Calling the intensity on the brighter row 100 per cent, that of the dimmer one is about 75 per cent, a difference of about 25 per cent. Calculations made by Hartridge (1922) show that in the image of a just resolvable single line the maximum intensity difference on two adjacent rows of cones is only about 10 per cent. The difference for the grating comes out to be over twice that value, indicating that perhaps the intensity difference in the image is not the limiting factor.

It is possible to test this by means of visual acuity measurements with three special gratings. One grating is similar to those used in that the opaque bars are equal in width to the spaces between them, and may be designated as  $1/2$ . The second grating has the same number of lines per centimeter as the first, but the width of the lines is only half and may be designated as  $1/4$ . The third grating has lines only half as wide again as those in the second grating and may be designated as  $1/8$ . The distribution of intensity in the retinal image of the first grating is as described in the preceding paragraph and drawn in Fig. 6B. The second grating at the same magnification gives an intensity distribution of the same periodicity as the first, but somewhat shallower, and symmetrical about the 75 per cent value instead of the 50 per cent value. The maximum intensity difference on two adjacent rows of cones is then only about 12 per cent. The third grating behaves similarly and gives only about a 6 per cent maximum difference in intensity for two adjacent rows. The expectation is then that the same maximum visual acuity should be achieved with the  $1/4$  as with the  $1/2$  grating, while the  $1/8$  grating should give a considerably reduced value. The average values for the maximum visual acuity for both observers with these three different gratings are with the  $1/2$ , 1.86, with the  $1/4$ , 1.84, and with the  $1/8$ , 1.75. It is therefore definitely established that the intensity discrimination of the retina in the "deteriorated" image is far from being a limiting factor in the resolving power of the eye for a grating.



The small, almost insignificant, lowering of the maximum visual acuity by means of the  $1/8$  grating led me to investigate further the basis of Hartridge's minimum value of a 10 per cent intensity difference in the image of a just resolvable single line. It was found that a  $5\ \mu$  wire placed in front of the field lens could be seen quite readily, while a  $4\ \mu$  wire could not. By means of a  $2.2\ \mu$  wire<sup>1</sup> mounted in the test object carriage the limiting values were found to be between  $4.2\ \mu$  and  $4.6\ \mu$ . These correspond to a visual angle of about 1 second of arc. The computation of the intensity distribution in the retinal image is quite inaccurate for such small separations, and the values of intensity difference on two adjacent rows of cones cannot be relied on to better than perhaps a factor of 2, but the value thus arrived at is between 1.5 and 2.0 per cent.

The second possible limiting factor concerns the pupil size, not as it affects the retinal image as a result of diffraction at the pupil and the aberrations of the dioptric mechanism of the eye, but in its relation to the amount of the diffraction spectra formed by the grating test object that it can transmit. Abbe (1873), in his work on the theory of microscope optics, demonstrated that in order for an image of a grating to be formed, the aperture of the objective must transmit the zero and at least one first order spectrum of the source. That this conclusion is not unique to microscope optics but applies to macroscopic optics as well is readily demonstrated by an experiment devised by A. B. Porter (1906) and described by Wood (1934).

Calculation showed that with a grating corresponding to a visual acuity of 1.8, a pupil 2 mm in diameter transmits the zero order spectrum and one entire first order spectrum up to about a wave length of  $630\ m\mu$ . A pupil of 3 mm diameter was therefore put into the apparatus, and the maximum visual acuity was determined as before. It increased about 15 per cent to a value of 2.12. In order to transmit up to  $\lambda 630\ m\mu$  of the first order spectrum of a grating corresponding to a visual acuity of 2.1, the pupil need be only 2.3 mm diameter. Such a pupil, actually measuring 2.35 mm, when put into

<sup>1</sup> The  $4$  and  $5\ \mu$  test wires were gilded quartz strings obtained from the Cambridge Instrument Co., Inc., of New York City and mounted between optical flats in air. The  $2.2\ \mu$  wire was a platinum Wollaston wire obtained from Baker and Co., Inc., of Newark, N. J., and mounted between a thin coverslip and an optical flat with Canada balsam.

the apparatus, yielded the same maximum visual acuity as did the 3 mm one, namely a value of 2.12. It is therefore clear that pupil size when it is less than 2.3 mm in diameter is the limiting factor in the resolving power of the eye for a grating.

The maximum visual acuity when the pupil size is not the limiting factor is 2.1. Since the grating is a repeated pattern, the smallest resolvable one must represent a spacing exactly equivalent to that of the elements in the retina. A smaller pattern would result in each cone being covered by a fraction of the light and dark stripes. A different situation obtains with a single object. There the maximum visual acuity may be much higher as it actually is here with the C, being 2.2 for E, L, S and over 2.5 for A, M, C and may even reach 30 times the grating value with a single stripe or line. This is analogous to the situation in the eye of the bee where the smallest grating resolvable is one that corresponds to the smallest ommatidial angle (Hecht and Wolf, 1929), while a single bar need be only about a quarter as wide to be seen (Buddenbrock, 1935) because a single row of ommatidia can detect an intensity change of about 25 per cent (Wolf, 1933*a, b*). Assuming the finest resolvable grating to correspond to the spacing of the retinal elements and taking the focal length of the eye to be 15.5 mm, the computed distance between adjacent rows of foveal cones comes out to be about  $2.1 \mu$ . Østerberg (1935), counting the number of cones in an area of 0.031 mm radius around the fovea, found the density of cones to be about 147,000 cones per sq. mm. Assuming the space occupied by a cone to be hexagonal, the distance between centers of adjacent cones comes out to be  $2.8 \mu$  and that between adjacent parallel rows of cones  $2.4 \mu$ . This value is an average over a relatively large area and is probably too large for the most central region as evidenced by the fact that Rochon-Duvigneaud (1906) found the cones in the central bundle of the retina to be from  $2.0$  to  $2.5 \mu$  in diameter.

The fact that the final value of the width of a row of cones is smaller than had been previously assumed in the calculation of the intensity difference on two adjacent rows of cones does not appreciably change the conclusions drawn. The previously assumed value was  $2.6 \mu$ . When a width of  $2.1 \mu$  is substituted, the percentage difference in the intensity rises from 25 per cent to 27 per cent.

It is of interest to note here that although the average diameter of

the pupil under high illumination is about 2.5 mm yet the smallest pupil diameter in the literature, that of P. R. in Reeves' (1918) paper, is only 1.9 mm. Thus the eye is built so that the two limiting factors, the pupil diameter and the cone separation, operate at nearly the same value.<sup>2</sup>

There are two other interesting points about the grating data. One is that the function of the para-foveal cones is not manifest in the measurements. Since the eye is presented by an extensive test field of  $4^\circ$ , one would expect the most sensitive elements to control the resolution at all times, resulting in a curve similar to that of Fig. 2. That such is not the case may be explained on the notion that a considerable homogeneous population of functional retinal elements is necessary for grating resolution. No such large homogeneous population of cones exists outside the fovea proper except perhaps in the periphery where the rods are more numerous and control the function of that area.

The other point has to do with the relative visual acuities for the two test objects at a given intensity. In Fig. 7 the curve for A. M. C. of Fig. 3 and the curve of Fig. 5 are drawn correctly spaced on their respective axes. It will be seen that below about 30 photons the grating gives superior visual acuity values, while at higher intensities the C is superior. Comparison of the rod curves and the first portion

<sup>2</sup> A question might arise in the experiments on the relation of pupil diameter to maximum visual acuity as to whether the observers' pupils were 2.35 mm in diameter and that the further increase in the artificial pupil produced no effect. That this is not so may be gathered from the following. When the apparatus is arranged for work with the grating, there are two overlapping images of the ground glass sources in the plane of the pupil. Because the grating decreases the intensity of the test field, that of the surround has to be decreased also. This is accomplished by means of a diaphragm on the ground glass source that supplied the surround illumination. The result is that whereas the image of the source from the test field always fills the pupil, that from the surround is always smaller. In order to facilitate work with the  $1/2$ ,  $1/4$ , and  $1/8$  gratings, this diaphragm is an iris that can be readily adjusted to give a brightness match for the two parts of the field under different circumstances. When the 3 mm pupil is substituted for the 2.35 mm, the test field becomes brighter than the surround for both observers, necessitating the opening of the iris controlling the surround brightness. This definitely shows that the natural pupils of both observers are wider than 2.35 mm.

of the cone curves for the two test objects shows that in order to achieve equal visual acuities about 10 times more light is necessary for the C than for the grating. This is not surprising when one considers that long before the opening in the C can be resolved the letter looks like an O, and that therefore the resolution of the O is more nearly comparable to the resolution of the lines in the grating. That the cone curves cross is the result of the fact that a higher maxi-

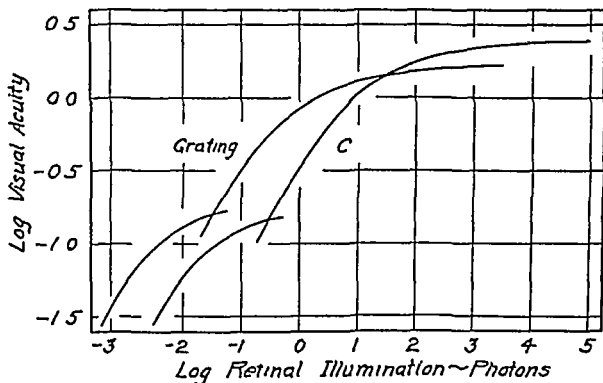


FIG 7 The curve for A M C of Fig 3 and the curve of Fig 4 showing the relative position on the axes of the two functions for the two different test objects

mum visual acuity is attainable with the C. Thus the maximum visual acuity with the grating is reached at about 1 logarithmic unit lower in intensity.

## VII

### *Comparison with Previous Data*

For comparison with previous data, I corrected Koenig's (1897) measurements with white light, to give relative retinal brightnesses. His intensity values were first converted into millilamberts. They were then multiplied by the photon factor (Troland, 1916), the pupil diameters being derived from the average found by Reeves (1918).

These values were then multiplied by a pupil efficiency factor derived from the data of Stiles and Crawford (1933). The data were then averaged in groups of five consecutive values, and plotted as log visual acuity against the logarithm of the retinal brightness in Fig. 8. The curves drawn in this figure are the same ones drawn through our own data.

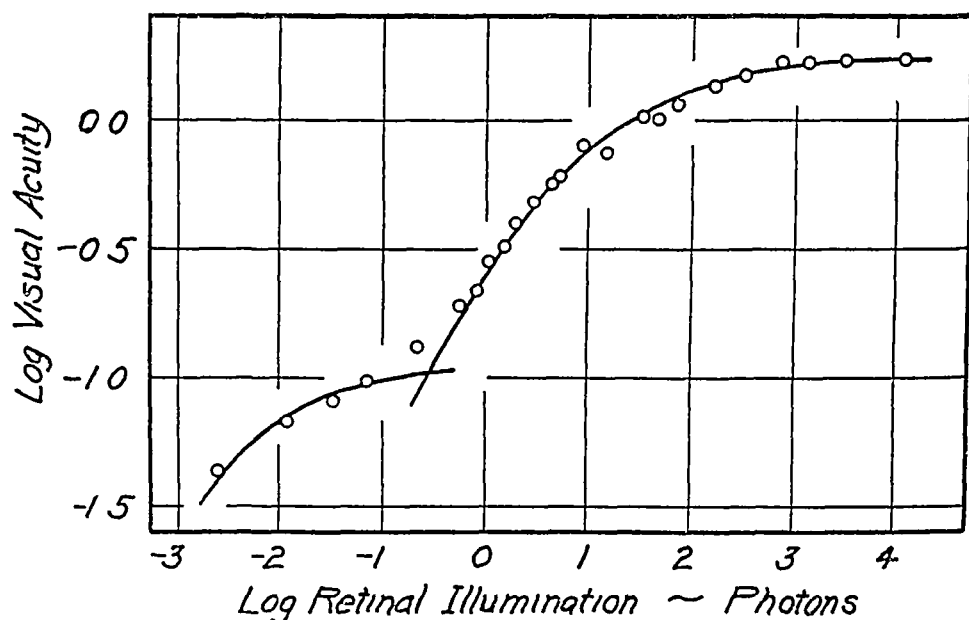


FIG. 8. The data of Koenig for white light with his intensity scale converted into retinal brightnesses by means of the data of Reeves on pupil area and the data of Stiles and Crawford on pupil efficiency and plotted as log visual acuity against  $\log I$ . The curve for the rods is of equation (3) and that for the cones of equation (2).

### VIII

#### Theory

The curves drawn through all the data represent two varieties of the stationary state equation developed by Hecht (1934) upon the most general considerations of the requirements of a photoreceptor process. Assuming a photosensitive material  $S$  which is changed by light to photoproducts  $P$ ,  $A$ ,  $B$ , and which is reformed again by a thermal reaction of some of these products, he derives the equation for the stationary state as

$$KI = x^n / (a - x)^m \quad (1)$$

where  $I$  is the light intensity,  $(a x)$  and  $x$  are the concentrations of sensitive material and photoproducts respectively,  $m$  and  $n$  are constants giving the orders of the photochemical and thermal reactions, and  $K$  is a constant

Setting the initial concentration  $a = 1$ , the values for  $KI$  for a series of values of  $x$  may be computed for the four cases where  $m = 1$ ,  $n = 1$ ,  $m = 2$ ,  $n = 1$ ,  $m = 1$ ,  $n = 2$ , and  $m = 2$ ,  $n = 2$ . By putting these values in the form of their logarithms, the nature of their dependence becomes independent of the values of the constants  $K$  and  $a$ , and may be plotted as  $\log KI$  versus  $\log x$  or  $\log v^n$  for each of the four cases. In this form the curves may be used to test any set of data that are plotted in the form of  $\log$  visual function against  $\log I$  by simple superposition. They then indicate the values of  $m$  and  $n$ . Such treatment has yielded gratifying results with the data of intensity discrimination (Hecht, 1935), flicker (Hecht and Schlaer, 1936, and Hecht and Smith, 1936), photosynthesis (Smith, 1937), instantaneous threshold (Hecht, 1937), and here, visual acuity.

The curve drawn through the cone data in this communication is that of equation (1) when  $m = 2$ ,  $n = 2$ , and where visual acuity is proportional to  $x^n$ . Equation (1) may then be written

$$KI = \frac{VA}{(VA_{\max}^{\frac{1}{2}} - VA^{\frac{1}{2}})^2} \quad (2)$$

This is identical with the form found to fit the data of intensity discrimination (Hecht, 1935) for the cones and differs from that found to fit the data of flicker only by the fact that flicker is proportional to  $x$  (Hecht and Smith, 1936).

Keeping visual acuity proportional to  $x^n$ , the data for the rods may be fitted about equally well either when  $m = 1$ ,  $n = 2$ , or when  $m = 2$ ,  $n = 1$ . The latter was chosen here to complete the similarity with the data of intensity discrimination, and may be written as

$$KI = \frac{VA}{(VA_{\max} - VA)^2} \quad (3)$$

## IX

### SUMMARY

1. An apparatus for measuring the visual acuity of the eye at different illuminations is described. The test object is continuously vari-

able in size and is presented at a fixed distance from the eye in the center of a  $30^\circ$  field. Observation of the field is through an artificial pupil. The maximum intensity obtainable is more than enough to cover the complete physiological range for the eye with white light though only 110 watts are consumed by the source. Means for varying the intensity over a range of  $1:10^{10}$  in small steps are provided.

2 The relation of visual acuity and illumination for two trained observers was measured, using two different types of test object, a broken circle and a grating. The measurements with both test objects show a break at a visual acuity of 0.16, all values below that being mediated by the rods and those above by the cones. The grating gives higher visual acuities at intensities less than about 30 photons and lower visual acuities above that. The maximum visual acuity attainable with the grating under the same conditions is about 30 per cent lower than that with the C. It is shown that the limiting factor in the resolution of the eye for the grating is the diameter of the pupil when it is less than 2.3 mm and the size of the central cones when the pupil is larger than that. The value of the diameter of the cone derived on that basis from the visual acuity data agrees with that derived from direct cone count in a unit of area.

3 The data for the cones made with both test objects are adequately described by one and the same form of the stationary state equation derived by Hecht for the photoreceptor system. This fact, together with certain considerations about the difference in the nature of the two test objects with regard to the resolvable area, leads to the conclusion that detail perception is a function of a distance rather than an area. All the data for the rods can likewise be described by another variety of the same equation, although the data are too fragmentary to make the choice of the form as certain as might be desired.

It is a pleasure to acknowledge the author's indebtedness to his professor and colleague, Dr. Selig Hecht, to whose friendly guidance throughout their many years of association the author owes much, and to his colleagues, Dr. Aurin M. Chase and Dr. Emil L. Smith who gave amply of their time and patience to make all the measurements here recorded, measurements which are perhaps the most arduous in the entire field of vision to make.

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## TRANSVERSE ELECTRIC IMPEDANCE OF NITELLA\*

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The large single cells of *Nitella* are very favorable material for the study of electrical characteristics. They can be found 5 cm or more long and about 0.5 mm in diameter. The single cell has a thin protoplasmic layer between the cellulose cell wall and the central vacuole. Presumably the bulk of the protoplasm is a relatively good electrical conductor but both the inner and outer protoplasmic surfaces are selectively permeable and each has a high electrical resistance and capacity. It has not been possible to differentiate the electrical properties of these two surfaces, so for simplicity, combined effects of both may be called the membrane resistance, capacity, or impedance.

Blinks (1930) measured the direct current resistances of living and dead cells between two electrodes separated different distances along the length of the cells, in air or a moist chamber. An average membrane resistance of 250,000 ohm cm<sup>2</sup> was found, and an approximate specific resistance of 87 ohm cm for the vacuolar sap is obtained from his results. From the time constants of the charge and discharge curves in this and a later paper (Blinks, 1936) he estimated a membrane capacity of 1.0  $\mu$ f/cm<sup>2</sup> which he suspected to be a polarization capacity.

In such longitudinal measurements, the paths of current flow are partly across the cell membrane and partly along the length of the cell, but they have been expressed with some success by the core conductor theory (Cremer, 1899, Hermann, 1905, Labes, 1932). Although the results of the theory have been applied to nerve, the postulates should be much better represented by *Nitella*. Blinks'

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data for the direct current resistance agree quite well with this model and lead with considerable certainty to substantially the values for membrane and sap resistance which he obtained by a simpler analysis. The membrane capacity may be calculated from the transients at the make and break of the measuring current, but this is somewhat complicated and unsatisfactory. The theory predicts that the form is given by the error integral which may differ from a simple exponential by no more than 10 per cent and this is a rather small margin in the analysis of oscillograph records.

As is well known, the transient and steady state properties are fundamentally identical (*cf.* Cole and Curtis, 1936). Since *Nitella* cells can be kept constant for hours at a time, considerably more accurate steady state alternating current impedance measurements over a range of frequencies should be possible.

The analysis of the longitudinal impedance of a single fiber (Cole and Curtis, 1936) may be extended and simplified when the electrodes are narrow and sufficiently widely separated, and the cell membrane has a finite resistance and a static capacity. In this case, from preliminary measurements at the Biological Laboratory, Cold Spring Harbor, Long Island, and Blinks' data, it was apparent that the maximum reactance would be at about 30 cycles per second. It would then be necessary to make measurements at 3 cycles per second and advantageous to go even lower. Although our apparatus is now equipped to go down to 30 cycles per second, it is not expedient at present to extend the range in this direction when an alternative type of measurement is available.

In transverse measurements, where the current flow between the two parallel electrodes is everywhere perpendicular to the axes of a suspension of parallel uniform fibers, the current distribution in and around the cells is relatively simple, the important frequencies are considerably higher and in a more convenient range than for the longitudinal measurements.

#### *Transverse Impedance Theory*

The impedance equation in this case (Bozler and Cole, 1935) is analogous to the Maxwell equation for a suspension of spheres,

$$\frac{1 - r_1/z}{1 + r_1/z} = \rho \frac{1 - r_1/z_2}{1 + r_1/z_2} \quad (1)$$

where  $r_1$  is the specific resistance of the suspending medium,  $z$ , the specific impedance of the suspension,  $z_1$  the equivalent specific impedance of the fibers, and  $p$  is their volume concentration. The circuit which is equivalent to the suspension consists of two resistances and an element whose impedance depends upon the frequency (Cole, 1928, 1932). In the derivation of this equation, a random distribution of the fibers is assumed (Cole and Curtis, 1936) but for *Nitella* it is desirable and possible to work with a single fiber. It is easily shown that the impedance of a single cylindrical cell symmetrically placed with respect to the electrodes is the same as a suspension of such cells in a rectangular array. This case has been worked out by Rayleigh (1895) who found equation (1) as an approximate solution. When the electrode separation is equal to the electrode width, the equivalent array is square and it can be shown that for a volume concentration of 0.5 or less a maximum error of 1 per cent will be made by the use of equation (1) rather than the second approximation given by Rayleigh. The theory for a measuring cell of rectangular cross section is more involved, but when the ratio of the dimensions is not far from unity the limiting volume concentration is only slightly reduced.

On the basis of the Rayleigh analysis, we shall use equation (1) and others which are derived either from it, or in a similar manner.

#### EXPERIMENTAL

*Nitella flexilis* was purchased from a tropical fish dealer and kept in aquaria with gravel and goldfish until used. Some time before measurements were to be made a suitable cell about 0.45 mm in diameter was cut loose from the adjoining cells, transferred to the measuring cell, and allowed to equilibrate. It was often found that slow, apparently spontaneous fluctuations of the low frequency impedance would occur for long periods with an otherwise normal resting cell.

Although this effect could result from a change of membrane resistance, it seems more probable that it was due to a salt transfer since it nearly disappeared when the external medium was flowed continually. The cells could be maintained in the measuring cell in good condition with vigorous protoplasmic streaming for as long a period as desired.

To obtain satisfactory transverse impedance measurements on a single *Nitella* cell it was necessary to use considerable care in the design and construction of the measuring cell. Cells of the type shown in Fig. 1, which were built up of glass with de Khotinsky cement were the most satisfactory.

The two electrodes of platinized platinum were 1.3 cm long and inlaid into

opposite sides of the groove into which the center portion of a *Nitella* cell was placed. This groove was 0.5 mm by 0.7 mm and 3 cm long, with depressions at each end which accommodated the cut ends of neighboring cells.

The top surface of the cell was polished flat and was covered with a flat glass plate after the *Nitella* cell was in place. The electrolytes used were flowed continually from a beaker into one end of the groove, through the measuring cell, and out at the other end by capillary glass siphons. It was possible to change the electrolyte concentration and reach the new equilibrium in about 5 minutes, and the impedance then remained stable and cell environment was known and constant.

The alternating current Wheatstone bridge used and the method of making the measurements is described in detail elsewhere (Cole and Curtis, 1937). The current through the measuring cell was so small that the impedance was independent of it. The impedance was measured as parallel resistance,  $R_p$ , and capacity,  $C_p$ , at sixteen frequencies from 30 cycles per second to 2.5 megacycles per second.

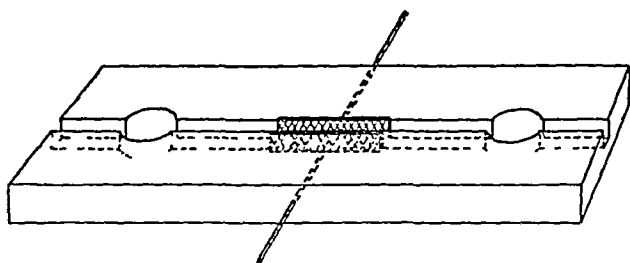


FIG. 1. Measuring cell for transverse impedance of a single *Nitella* cell.

At the lower frequencies it was necessary to make corrections, which were usually small, for the polarization impedance of electrodes.

The data were calculated<sup>1</sup> and plotted with series resistance,  $R_s$ , and reactance,  $X_s$ , as abscissae and ordinates to give the impedance locus.

### Data and Calculation

When a *Nitella* cell, suspended in an electrolyte, is equivalent to a circuit containing two resistances and a variable impedance element, the impedance locus is a circular arc, and the phase angle of the variable impedance element is half the angle subtended by lines drawn

<sup>1</sup> The formulae are

$$R_s = \frac{R_p}{1 + (R_p C_p \omega)^2}, \quad X_s = \frac{R_p^2 C_p \omega}{1 + (R_p C_p \omega)^2}$$

where  $\omega$  is 2 $\pi$  times the frequency in cycles per second.

from the two intersections of this curve with the resistance axis to the center of the circle (Cole, 1928) It is seen by the data for a *Nitella* cell which is plotted in Fig 2, that such a circuit is a close approximation over most of the frequency range The phase angle in this case is  $77^\circ$  and the average value which has been found for *Nitella* is  $80^\circ \pm 4^\circ$

If a *Nitella* cell consists simply of a non conducting membrane surrounding an electrolyte, the volume concentration,  $\rho$ , of the cell sus

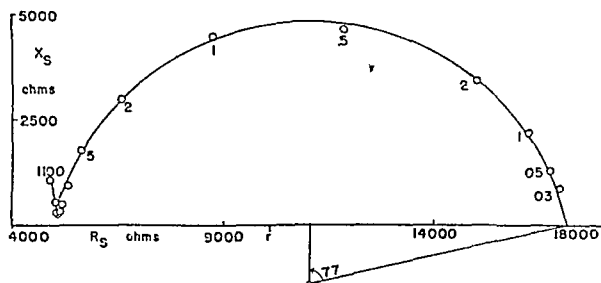


FIG 2 Impedance locus series resistance  $R_s$  vs series reactance  $X_s$ , for a *Nitella* cell in tap water Frequencies are given in kilocycles per second

pending in an electrolyte of resistivity  $r_1$  is given by (Bozler and Cole, 1935)

$$\frac{1 - r_1/r_0}{1 + r_1/r_0} = \rho \quad (2)$$

where  $r_0$  is the low frequency resistivity of the suspension When this equation was applied to the data of *Nitella*, however, it was found that computed values of  $\rho$  were always markedly lower than those obtained from the inner diameter of the cell wall The most obvious explanation of this would be that the membrane is not non conducting, but has a finite and measurable resistance On this hypothesis the membrane resistance would have an improbable value of about 1 ohm cm which would depend on the medium There is, however, another explanation which is worth investigating

*Cellulose and Membrane Resistance*

The *Nitella* cell is surrounded by a layer of cellulose which has been thought of as being completely permeable and therefore having a resistivity equal or at least proportional to the resistivity of the suspending medium. If, however, the cellulose wall had a resistivity independent of the suspending medium, it would be possible to explain the discrepancy in volume concentration measurements. To test this hypothesis, and at the same time obtain the other electrical constants of the cell, runs were taken in the following manner. A cell which had been carefully selected for measurement was placed in the measuring cell and tap water circulated around it. When it had become equilibrated, the parallel resistance and capacity were measured over the entire frequency range. Without disturbing the cell, 1 per cent sea water was substituted for the tap water, the procedure repeated, and again for 10 per cent sea water. The *Nitella* cell was then removed and its ends cut off. A glass rod was drawn out to approximately the internal diameter of the cellulose wall and several feet long. Such rods are always slightly tapered, and, starting at the small end, this cellulose tube was pushed as far as possible under water toward the large end. The rod was then broken at each end of the tube, placed in the measuring cell, and complete frequency runs taken for the three electrolytes. Then that portion of the tube between the electrodes was carefully cut away from the glass rod and the procedure repeated. Finally the glass rod was removed and the process repeated on the three electrolytes alone. The entire series could be completed in about 10 hours.

Table I gives specific resistances obtained from such a run. Those for the living cell are the extrapolated zero frequency values. The resistances for the suspending medium and for the uncovered glass rod were independent of the frequency and the values for the covered glass rod were nearly so. It will be noticed that for the high resistance medium the resistance with the glass rod is lower when it has the cellulose wall over it than when it is by itself, and that for the low resistance medium the opposite is the case. This in itself shows that the resistivity of the cellulose wall is neither the same as the resistivity of the suspending fluid nor even proportional to it. Comparing the resistances for the live cell and the glass-filled cell, we notice that the

former are higher in every case. This might be taken as an indication that the membrane and cell interior had a higher equivalent resistance than glass, but it seems more probable that either the cellulose fits more tightly over the living cell than over the glass rod, or better still that volume concentration,  $\rho$ , is greater in the live cell due to the turgor pressure. But from these data it is possible to assign a value to the resistivity of the cellulose.

By a method similar to that used previously for muscle (Cole and Curtis, 1936), assuming that the thickness of the cellulose,  $\delta$ , is small

TABLE I  
*Specific Resistances in Ohm Cm As Explained in Text*

	Tap water	1 per cent sea water	10 per cent sea water
Suspending medium	9 240	1 578	180
Live cell	15 500	4 000	545
Glass rod			
Cellulose on	13 670	3 300	440
Cellulose off	20 620	3 520	397.5

in comparison to the radius,  $a$ , of the glass rod, the following formula for the cellulose resistivity,  $r_4$ , may be derived,

$$r_4 = r_1 \frac{(1 + 2\delta/a)(r'_0 - r_1)(r_0 + r_1) + (r_0 - r_1)(r'_0 + r_1)}{(1 + 2\delta/a)(r_0 - r_1)(r_0 + r_1) - (r_0 - r_1)(r'_0 + r_1)} \frac{a}{a + \delta}$$

where  $r_0$  and  $r'_0$  are the low frequency resistivities of the glass rod suspension with and without the cellulose wall respectively. Since we must consider both  $r_4$  and  $\delta$  as unknowns, measurements in a single medium can only define a relation between them. There is then a single value of  $r_4$  corresponding to each possible value of  $\delta$  which gives a curve when  $r_4$  is plotted against  $\delta$ . For the different media, the three curves are found as shown in Fig. 3 and if the cellulose resistance and thickness are independent of the medium, these curves should intersect at a single point. The center of the circle tangent to the three curves gives 1150 ohm cm for the resistance and  $12.8 \mu$  for the thickness which best fit all three sets of data. Thus the cellulose resistance was so slightly affected when the conductivity of the medium was changed by a factor of more than 50, that we may



consider it to be substantially independent of these media. Direct measurements indicated that the thickness of the cellulose wall was about  $10\mu$ . Although we have not taken accurate direct thickness measurements, those that we have agree quite well with the computed values and give about  $10\mu$  for the mature cells. In one young cell the thickness obtained from the electrical measurements was  $4\mu$  while the directly measured value was  $5\mu$ . This general agreement may be

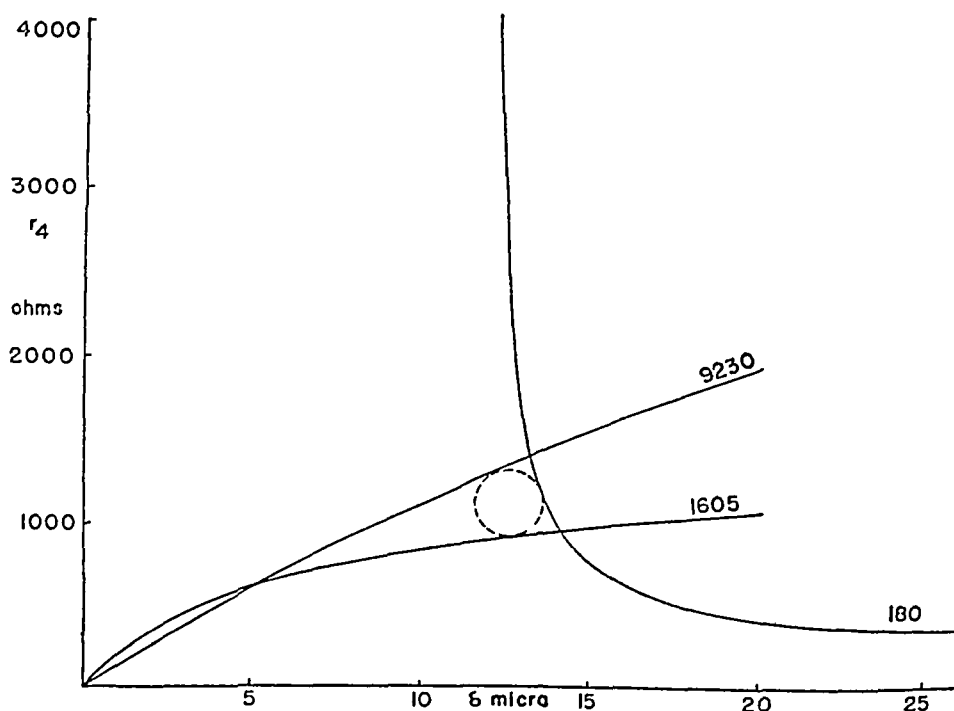


FIG 3 Thickness  $\delta$  vs specific resistance of cellulose sheath  $r_4$  from measurements in three media having specific resistances indicated

taken as further support for the assumption of a constant cellulose wall resistance

Turning now to the low frequency measurements of the intact cell, we prefer to delay making an assumption of the membrane resistivity and to calculate the equivalent resistivity,  $r'_2$ , which a cell representing a volume concentration,  $\rho$ , would have if it were a homogeneous conductor. In Fig 4 the same type of graphical simultaneous solution used above gives values of  $r'_2 = 27,000$  ohm cm and  $\rho = 50.2$  per cent

Since a value of  $\rho = 48$  per cent was estimated from the over all cell diameter, we have some reason to consider the value of  $r'$  seriously. It may be noted that the intercepts of the curves of Fig 4 on the horizontal axis are the values of volume concentration which are calculated by equation (2) if the cellulose is ignored and the protoplasmic surface assumed to be non conducting. Also the volume

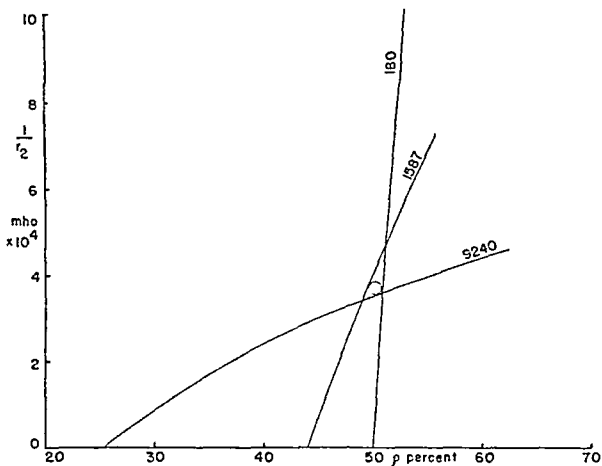


FIG 4 Volume concentration  $\rho$  vs equivalent low frequency conductance  $1/r_2'$  of *Nitella* from low frequency measurements in three media having specific resistances indicated

concentration of the glass filled cellulose was found to be 42 per cent, which suggests that the difference between its resistance here and when it is covering the live cell is due to turgor

If we now assume that the membrane is, within the limits of experimental accuracy, non conducting, we have (Cole and Curtis, 1936),

$$\frac{1 - r_4/r_2'}{1 + r_4/r_2} = \frac{a_1^2}{a_2^2}$$

where  $r_4$  is the resistivity of the cellulose wall and  $a_1$  and  $a_2$  are the inner and outer radii respectively. Substituting the values of  $r_2$  above and  $r_4$  from the glass rod data, we find  $\delta = a_2 - a_1 = 8.5\mu$ , which is somewhat less than the previous value. But we may say that within our experimental limits, the membrane may be considered non-conducting.

### *Membrane Capacity and Internal Resistance*

In previous work, the membrane capacities have been calculated at the characteristic frequency, for which the series reactance is a maximum, or better, where the series resistance takes on the mean value of the extrapolated zero and infinite frequency resistances (see Cole and Curtis, 1936). In the case of *Nitella*, the cellulose is a nuisance in that we have been unable to evolve any but elaborate, and tedious, theory and calculations for the determination of the capacity and internal resistance.

The characteristic frequencies range from 0.63 kilocycles per second for the dilute medium to about 16 kilocycles per second for the most concentrated, and in spite of the difficulties, it is rather remarkable that the capacities are constant at  $0.94\mu\text{f}/\text{cm}^2$  to within  $\pm 10$  per cent at the respective frequencies for the different media.

We have not been so fortunate in the determination of the internal specific resistance in the living cell. By a procedure consistent with that used in the calculation of the capacities, we obtain from 95 ohm cm for the concentrated media to 1800 ohm cm for the dilute media. It was to be expected that this resistivity would be relatively independent of the medium. Measurements on sap extracted from cells which had been soaking in the three media gave 58 ohm cm  $\pm 8$  per cent, with no correlation with the medium resistance.

The internal resistances were calculated on the basis of the only infinite frequency extrapolations which could be made, as is shown in Fig. 2, but it is seen that there are indications of structures with a characteristic frequency above the present range. These probably cannot be invoked to extricate us from our present resistance difficulties, but preliminary measurements suggest that they may be the chloroplasts.

### *Chloroplasts*

In order to investigate the effect of the chloroplasts, runs were taken on a cell with both ends cut off in the three media, and again after the chloroplasts have been washed out. The results were not particularly satisfactory because the change in resistance with frequency was very small, but it did show that the chloroplasts (or perhaps pieces of protoplasm adhering to the cell wall) have a resistance and capacity which is a function of the frequency. An effort was made to isolate the chloroplasts and measure their electrical properties independently in a measuring cell which holds 0.005 cc of suspension. A satisfactory suspension of chloroplasts has not yet been obtained, but preliminary results agree with the dead cell data, and indicate that the impedance properties of the chloroplasts are similar to those of living cells. However, more definite conclusions will have to await further measurements.

### DISCUSSION

The assumptions that *Nitella* has a non conducting membrane, and that the cellulose wall has a resistance which is independent of the suspending medium seem to have worked out rather well. The data are consistent with them and furthermore lead to approximately correct values of the wall thickness. Blinks' longitudinal data on dead cells which have been filled with air are interesting in this regard. Assuming that the walls of his cells were  $10\mu$  thick, we compute a value of 850 ohm cm for the specific resistance of the cellulose, which agrees quite well with our values. It seems premature to speculate about the nature of the conductance in cellulose on the basis of the present measurements, but we hope in the near future to check this behavior of cellulose in material taken from other sources.

The average membrane capacity of  $0.94 \mu\text{f}/\text{cm}^2$  which was obtained on these assumptions seems reasonable. It is very nearly the same as has previously been found for a wide variety of different cells, and confirms Blinks' estimate of  $1 \mu\text{f}/\text{cm}^2$ . The average phase angle of this capacity is  $80^\circ$  which indicates that it is a polarization impedance only slightly removed from a static capacity.

It was suggested in the work on nerve and muscle (Cole and Curtis, 1936) that an equivalent polarization impedance element could result

from a statistical distribution of membrane static capacities and cell diameters. Further calculations on this postulate have indicated that it alone is probably not adequate, and it has not yet been possible to check it for nerve or muscle by measurements on single fibers. The present *Nitella* polarization impedance might be interpreted as a small variation of a membrane static capacity from point to point over the surface of the cell and while this is not an unreasonable picture, it is not particularly attractive.

The value of the resistivity of the sap which was measured directly on sap squeezed from the cells is probably substantially correct. In a similar manner, Hoagland and Davis (1923) obtained 89 ohm cm. Calculations made from Blinks' (1930) data for dead cells give a value of 87 ohm cm, and the same value is obtained from his transient measurements on live cells taken at very short times. Preliminary data on chloroplasts show that they undoubtedly play some part in the calculations of the resistivity of the sap from our high frequency measurements on the live cell, and on the face of it, it does not seem as though they could account for the large discrepancies observed. However, one could assume that they were packed very tightly around the inside of the living cell, being held firmly in place by the turgor pressure. Then if the turgor pressure were released by placing the cells in a strong salt solution or by killing them, the chloroplasts would not be packed nearly so tightly and would not exert nearly as much influence on the measurements. Since Blinks took his measurements with the current flow primarily parallel to the cell axis instead of perpendicular to it, he would not be troubled by this postulated phenomenon. This would explain why his results on the living cell check the direct measurements and ours do not, and explain why our measurements taken in concentrated media come quite close to the correct value whereas the ones taken in dilute media do not. However, in following out this reasoning one runs into difficulties with the capacity values, so that further speculation can be indulged in profitably only after the impedance properties of chloroplasts are better known.

It is interesting to observe that transverse measurements cannot hope to detect a membrane resistance as high as that observed by Blinks in longitudinal measurements. The problem is essentially

that of measuring the volume enclosed by the membrane with an accuracy which is impractical at present, but has been discussed in connection with marine egg measurements (Cole, 1937)

#### SUMMARY

Alternating current measurements have been taken on single *Nitella* cells over a frequency range from 30 to 2,500,000 cycles per second with the current flow perpendicular to the axis of the cell. The measuring cells were so constructed that electrolytes of any desired concentration could be circulated during the course of the measurements. The cellulose wall which surrounds the cell is found to play an important part in the interpretation of the results obtained. In a mature cell, this cellulose has a specific resistance of about 1000 ohm cm which is independent of the medium in which the cell is suspended. The thickness of the wall is computed to be about  $10\ \mu$ . The cell membrane is found to be virtually non conducting, and to have a capacity of  $0.94\ \mu\text{f}/\text{cm} \pm 10$  per cent and a phase angle of  $80^\circ \pm 4^\circ$ .

The specific resistances of the sap were difficult to compute from data on living cells and were unsatisfactory because they were very much dependent upon the medium, while measurements on extracted sap gave  $58\ \text{ohm cm} \pm 8$  per cent which was independent of the medium. There are indications that the chloroplasts have impedance properties similar to those of living cells.

We are indebted to Mr J M Spencer for his assistance

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# INTENSITY AND CRITICAL FREQUENCY FOR VISUAL FLICKER

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## I

For the investigation of visual flicker in fishes, amphibians, and certain insects we have used<sup>1</sup> the procedure of exposing an animal in a space surrounded by a rotating cylinder upon which there alternate transparent and opaque vertical stripes. Light of adjustable, measured intensity is reflected through the transparent stripes. The critical illumination,  $I_c$ , is obtained which, for a given speed of rotation of the cylinder, results in forced reactions of orientation and movement by the organism tested. Alternatively, by adjustment of the speed of rotation of the cylinder, the critical flash frequency is obtained by test which, for a given intensity of illumination in a flash, forces the same response. The critical flash frequency,  $F$ , is given by the number of flashes passing a given point at the circumference of the cylinder in a unit time.

The relationships between  $F$  and the mean values of  $I_c$  ( $= I_m$ ), and between  $I$  and the mean values of  $F_c$  ( $= F_m$ ) have been discussed<sup>1</sup> from the standpoint that the response to the visual flicker, and to the direction of its movement, is homologous to the recognition of flicker in human visual experiments. For this position it can be said, in the first place, that the curves thus obtained for the relationship between  $F$  and  $I$  have the same general properties in various organisms<sup>1</sup> as are found for marginal suppression of human visual flicker, further, that the curve obtained with the bee<sup>2</sup> by means of a quite different technique<sup>2</sup> has again the same general properties.

<sup>1</sup> Wolf and Zerrahn Wolf 1935-36 Crozier, 1935-36, Crozier, Wolf, and Zerrahn Wolf, 1936-37 *a b c, d* 1937-38 *a*

<sup>2</sup> Wolf 1934-35



There arise in this connection certain special questions for the theory of critical flicker frequency. We are not now required to enter upon a detailed discussion of these particular questions, but their significance must be referred to, they will be more elaborately considered in subsequent papers.<sup>3</sup> One point we attempt to deal with now, because it is important for the general theoretical treatment the question, namely, as to whether a valid homology may be held to obtain with respect to the flicker response data provided by lower animals on the one hand, as secured by the method outlined, and on the other hand the data of critical illumination as a function of flicker frequency for the human observer.

It is clear that the *general* form of the functional interrelationship of  $F$  and  $I$ , as it has been derived from different experiments, is the same for our measurements with fishes as in the case of the human observations.<sup>4</sup> Both exhibit the composite character attributable to the duplex constitution of the vertebrate retina,<sup>5</sup> and both (at least certainly, in really comparable data, for the cone portion of the curve) are adequately described by a probability integral in which  $\log I$  is the abscissa. The human data, however, have been based essentially upon flicker *fusion*, while the data with lower animals are based upon threshold response signifying marginal *recognition* of flicker. It has been desirable to demonstrate experimentally that the two kinds of observations are comparable. By essentially comparable we of course mean that the form of the functional connection between  $F$  and  $I$  may be legitimately used to develop and to test the theoretical significance of such data for the interpretation of visual responses, with confidence that in the two sorts of cases the data have the same kind of meaning. A direct proof of the adequacy of this position is to be obtained by placing the eye of the human observer in the position occupied in our apparatus by a fish or insect larva. If under these conditions a technique and procedure essentially identical with that employed for other animals provides for human responses a flicker recognition curve basically identical with that known to be characteristic for flicker fusion in human visual reactions, the required demonstration is

<sup>3</sup> Cf. Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b, c*

<sup>4</sup> Crozier, 1937

<sup>5</sup> Hecht, 1937, Hecht and Verrijp, 1933-34 *a, b*, Hecht and Smith, 1935-36

manifestly provided. In other words, it may be held with some confidence that what the lower animals are responding to is properly labelled "flicker."

The simple arrangement satisfying these conditions is illustrated diagrammatically in Fig 1. The details of procedure have been varied in a number of ways, in different experiments. In tests with fishes and the like the practice has uniformly been<sup>1</sup> to obtain three readings of  $I_c$  or of  $F_c$  at one time upon each of ten (or twelve) individuals, the same individuals being used in securing the entire set of measurements for an  $F-I$  curve. Tests must be applied to insure that the individuals thus treated together are objectively a homogeneous<sup>6</sup> group. Homogeneity thus defined means that the individuals concerned are essentially equivalent, and in a long series of observations could be interchanged. The average value of  $I$  or of  $I$  for an individual =  $I_1$ , or  $I_1$ , the mean of these numbers for the homogeneous group of individuals =  $F_m$ , or  $I_m$  respectively. The reasons for this procedure have been (1) that it reduces the influence of adventitious, irrelevant errors of observation and (2) that analysis of the variations of the individual magnitudes of  $I_1$  (or  $F_1$ ) in successive tests shows<sup>7</sup> that they correspond to natural fluctuations of excitability (responsiveness) in any one animal.

In experiments of this type<sup>1</sup> the determinations of  $I$  are made by slowly increasing the intensity of the flashing light until response of the organism tested is obtained at  $I_c$ , the same end point is also approached, in other trials, by fixing the intensity and slowly reducing the flash frequency until the same end point is signalled ( $F_c$ ). Reasons have been discussed<sup>8</sup> (and tested<sup>9</sup>) for the contention that, under the conditions described,  $F_m - I$  and  $I_m - F$  curves cannot be expected to agree but must be found to differ in a predictable way. If observations of  $F_1$  and  $I_1$  (as already defined) are made at intervals upon a single organism this expectation is not in question, when the method of signaling excitation during increase of  $I$  or decrease of  $F$  is being used. It is in point if the method of careful back and forth

<sup>6</sup> Crozier 1936

<sup>7</sup> Cf. Crozier, Wolf, and Zerrahn Wolf 1936-37 a, b, c, d, 1937-38 a

<sup>8</sup> Crozier, 1935-36

<sup>9</sup> Crozier, Wolf, and Zerrahn Wolf, 1936-37 a, b

adjustment to an apparent critical point is the procedure employed—as published data unmistakably signify<sup>10</sup> and as our own observations unequivocally demonstrate. The importance of these considerations is of two sorts. It has a bearing upon the process whereby theoretical interpretations are tested by the adjustment of curves to the observations, it has a more direct interest for the understanding of the way in which variability of responsiveness is involved in the determination of  $F_c$  or  $I_c$ .<sup>11</sup>

The procedure in the present experiments has been varied in order to throw light upon certain of these points, which will be amplified in subsequent papers. The technique has some manifest disadvantages for the precise investigation of the human visual response to flicker, but it has compensating advantages. We may repeat that our primary purpose in these experiments was to demonstrate whether the curves of "response to visual flicker" as we have analyzed them in the case of lower organisms<sup>1</sup> are essentially homologous to the data of human visual flicker. We have therefore employed the same apparatus for the determination of both. In the method used,<sup>1</sup> however, the opaque stripes necessarily reflect a certain percentage of the illumination used. Whether in our tests with the human eye this fact introduces complications of interpretation could be decided only by means of an elaborate series of experiments designed to make clear the influence of area, location, and form of test patch, and of wave length of light, upon the form of the  $F - I$  curve, as well as of the fundamentally significant effect of modifications of the proportion of light time to dark time ( $t_L/t_D$ ) in a flicker cycle. Our experiments with fishes and with insects<sup>12</sup> have shown that, with the apparatus and procedure we here employ, (1) the *form* of the  $F - I$  curve is not affected by changes of ( $t_L/t_D$ ) and (2) that the change of  $F_c$  as a function of  $I$  is not that which appears when reflection from the blackened surface of a sector disc is a factor, but is on the contrary that obtained<sup>13</sup> when flicker is produced by interruption of a beam of light. Since in any case we are concerned with the *form* of the function, these interest-

<sup>10</sup> Hecht, Schlaer, and Smith, 1935, Hecht and Verrijp, 1933-34 a, cf. Crozier, Wolf, and Zerrahn-Wolf, 1936-37 a

<sup>11</sup> Cf. Crozier, 1936, Crozier and Holway, 1937

<sup>12</sup> Crozier, Wolf, and Zerrahn-Wolf, 1937-38 b, c, 1937

<sup>13</sup> Cf. Piéron, 1922, 1936

ing and theoretically important features of the case may therefore be ignored

In one set of experiments we have, with each observer, systematically obtained ten successive determinations of  $I$  or of  $F_c$  at each of a number of levels of  $F$  or of  $I$  respectively, on different dates. The eye was adequately adapted to darkness, and then to the level of  $I$  being used, preliminary practice then assured absence of drift in successive readings. The corresponding mean values,  $I_m$  or  $F_m$ , obviously have mainly the status of  $I_1$  or  $F_1$  in our tests with other organisms<sup>1</sup>. It is clearly not to be expected that systematic differences should be apparent between  $F_m$  and  $I_m$  at equivalent levels, unless these values are obtained (with a given observer) *at the same time*. The variation in  $I_c$  or in  $F_c$  is of course still expected<sup>1</sup> to throw light upon the interpretation of the mechanism initiating the index response. It will be shown that the variation of  $F_c$  and of  $I$  respectively follows different rules, which are severally consistent with the indications already provided by experiments with other organisms<sup>1</sup>.

In another series of tests the procedure was adjusted to correspond more nearly with that followed in the experiments with fishes and insects. Three readings of  $F_c$  were obtained on each of five occasions at each of a number of intensities. The interpretation of these data will be discussed subsequently.

It has already been remarked that the instrumental procedure here used has certain theoretical deficiencies. One of its compensating advantages is that quite low flicker frequencies can be employed and maintained with relatively high precision. This has made it possible to extend the flicker curve to  $F \approx 1/\text{sec}$  or below, and to demonstrate that its form in the region  $< F = 10$  is like that observed with teleosts. A disadvantage is that very high intensities ( $\log I$  for a flash  $> 2.5$  millilamberts) cannot be obtained or varied with adequate precision. A knowledge of the kinds of complication legitimately to be expected in the flicker curve for a vertebrate, however, makes possible a useful analysis of the form of the function without an exact knowledge of the maximum to which the  $F - \log I$  curve rises<sup>14</sup>.

<sup>14</sup> In any case, the observable value of the maximum may be adventitiously affected by decline phenomena influenced by 'glare' and other factors in such a way as to be of minor analytical significance. Moreover at very high values of  $F$  the precision of the apparatus is deficient in comparison with that obtaining (section II) at lower flash frequencies.

## II

By means of two prisms and a short-focus telescope the line of sight from outside the rotating striped cylinder was focused upon the interior surface (Fig 1) In effect, the human eye was thus put in the position of an animal located in an

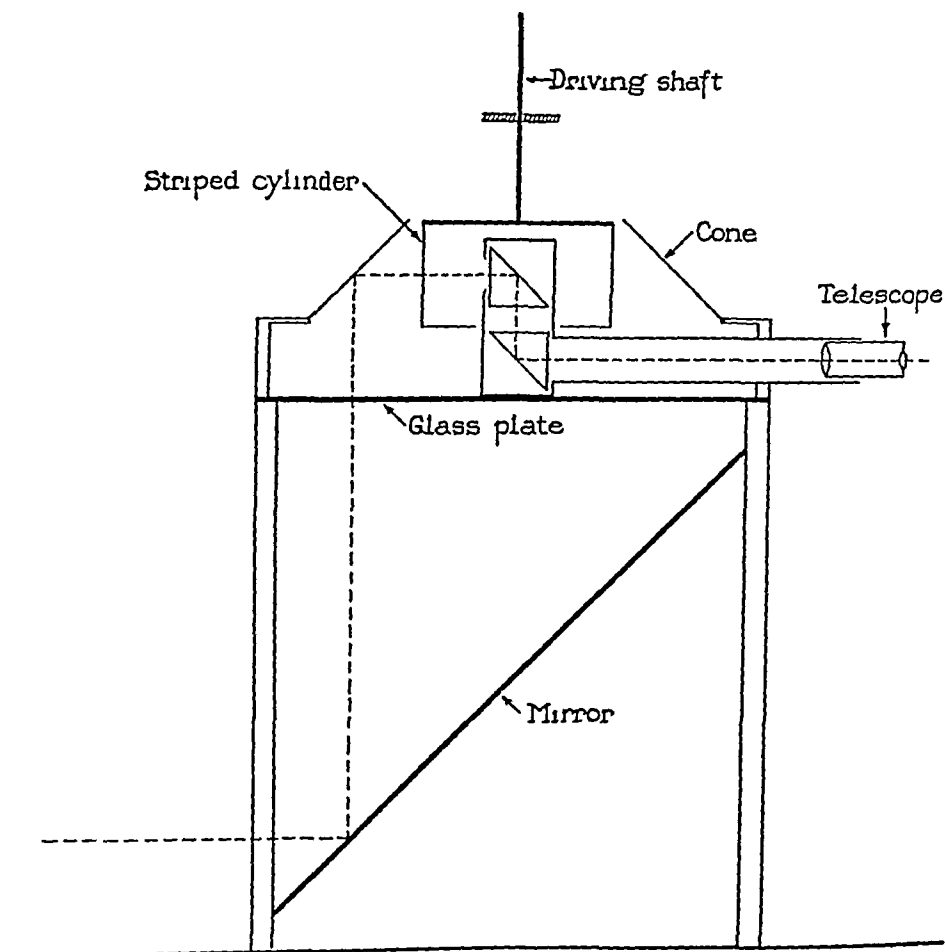


FIG 1 The rotating cylinder device<sup>1</sup> (diagrammatic) as modified for measurements of human response to flicker. The apparatus is essentially the same as used for tests with lower organisms. By means of a set of two prisms and a telescope the observer views the striped cylinder in the position of an animal placed inside the striped screen. The visual field is square, with a visual angle of  $14.3^\circ$  on an edge.

aquarium inside the cylinder,<sup>1</sup> with the difference that only a patch of the surface was now exposed to view. Square diaphragms in the viewing tube (Fig 1) delimited a square field with vertical edges parallel to the edges of the opaque

stripes on the cylinder. The visual angle of the field was about  $14.3^\circ$  on an edge. A large field (with central fixation) was employed partly in order to test a possible influence of the number of divisions between light and dark areas, using cylinders with different numbers of equally spaced stripes, and thus of different stripe widths, it was found that there was (within the range used) no effect due to the number of dark bars in the field.

A square field has disadvantages as compared with a circular one since flicker at the corners may be apparent at an intensity below that which is critical for the center of the field. It is also clear that the method of transillumination of the cylinder allows light to be reflected from the inner surface of the opaque stripes. The complications arising in this way are of no consequence for our immediate problem since the proportion of light time to dark time in a flash cycle was kept constant at 1 and we are concerned only with the form and structure of the curve.

Observations were made by the method used with lower animals. With a fixed illumination the speed of rotation of the cylinder was slowly reduced from a high level until the observer signalled the operator that flicker was recognized. In the converse test, with a fixed flash frequency the intensity was slowly increased from zero until flicker was signalled.

The intensities were obtained from calibration curves giving measured illumination at the eye piece of the telescope as a function of diaphragm opening.<sup>1</sup> No attempt was made to correct for pupil area. Cylinder revolution speeds were measured by means of millivoltmeter readings from a magneto geared to the cylinder driving shaft.<sup>1</sup> Speeds were adjusted with coarse and fine rheostats in the motor circuit.

Observations were taken in groups of ten successive determinations of the flicker response point ( $I$  or  $F_s$ ). The order of succession of levels of  $F$ ,  $I$  was so arranged as to reveal any drift with time or practice. Fatigue of the observer was avoided as much as possible. In a few cases successive determinations of  $F_m$  and  $I_m$  were made at the same level.

Data secured with two observers, after preliminary practice on a number of days, are set out in Fig. 2. The curves are of the same type in the two cases, but exhibit interesting differences. The general form is that already found by other observers using rotating sector devices.<sup>10-5</sup> There can be little doubt that the form of the flicker function has not been influenced by the fact that the opaque bars are moving across the field. The end point used in the observations of Fig. 2 was a flutter of the field, a slightly higher intensity is required at each  $F$  to give definite response to the direction of movement of the bars, but there is no doubt that curves obtained with this end point would have the same form.

The area of our square  $14.3^\circ$  field is a little less than that of the  $19^\circ$  circular field used by Hecht and Smith (1935-36). The curves have very much the same morphology: the maxima for rod and cone parts are about the same, and even the indication of a slight hump near  $F = 35$  is also apparent in all these curves, the slopes of the practically rectilinear parts of the graphs are about 10.4 for E W, 12.0 for

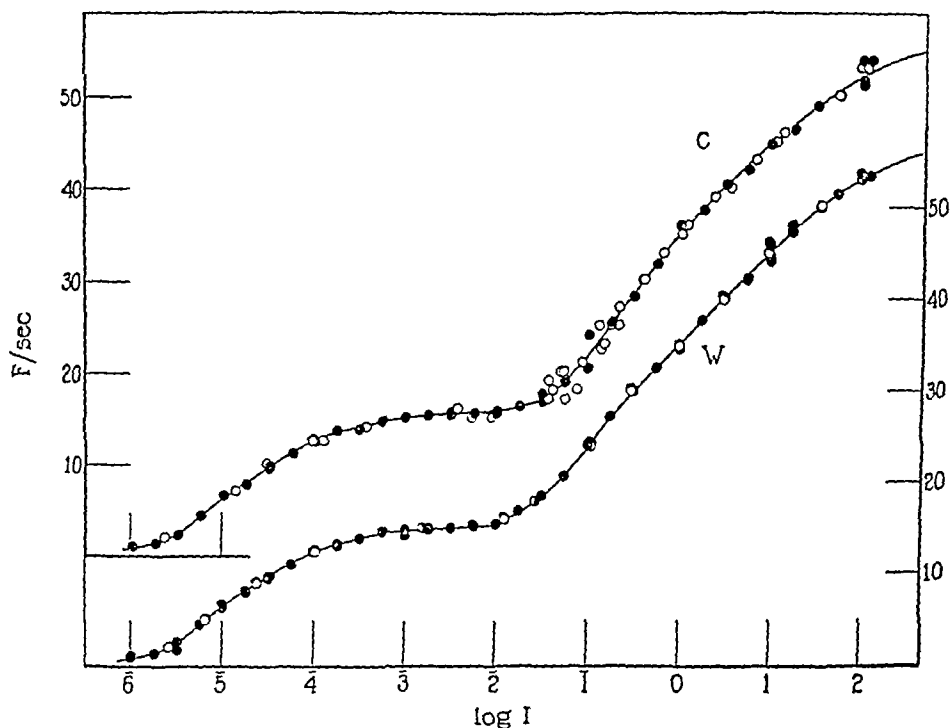


FIG. 2. The relation between critical flash frequency  $F$  and critical intensity  $I$  for human response to flicker. Solid circle,  $F_m$  at fixed intensities  $I$ , open circle,  $I_m$  at fixed  $F$ .

Upper curve, left eye of W J C, lower, left eye of E W ( $F$  scale at the right).

The data shown are from homogeneous sets of measurements taken on a series of successive days, after considerable practice. Each plotted point is the mean of ten readings. Total number of observations: W J C, 1,220; E W, 880.

W J C, and 12—for E L S, the separation of the rod and cone curve inflection points is identical, 3.45 log units in all three cases. These facts, together with others subsequently discussed, show that the curves in Fig. 2, obtained by the rotating cylinder method, represent the typical properties of the critical flicker mechanism for human visual excitation.

The corresponding curves for fishes<sup>1</sup> are very similar. They cover about the same intensity range, but (especially with allowance for the effect of temperature<sup>7</sup>) are on the whole situated at lower levels of intensity, despite the fact that the signalling response involves reaction to the direction of movement of the stripes, a further element in this case is the fact that the whole retina is excited by a field of unlimited extent. The cone curves for various fishes are usually steeper. The rod curve for fishes is of lower maximum  $F$ , and at a definitely lower intensity as a whole. Thus the extent of separation of the inflections in rod and in cone portions is definitely greater (about 5 log units) than in the human curves. The latter fact is of special interest for the analysis of the composite graph (section IV).

### III

1 The variations in successive determinations of the flicker end point follow the general rules already obtained for motor reaction to photic flicker in lower animals.<sup>1</sup> The experiments were not specifically designed to examine all the properties of this comparison. In a later connection we expect to do this more critically, with apparatus designed for use with the human eye. One difference in the nature of the data is to be noted: the properties of the scatter of  $I_1$  and of  $F_1$  were, in the latter cases, obtained from the values of  $I_1$  or  $F_1$  as averages of three measurements each on each of a set of individuals, the several individuals being demonstrated to be equivalent. The reasons for this procedure have been described.<sup>1</sup> In the present instance,  $PE_{11}$  and  $PE_{12}$  are based upon a set of ten successive readings with one individual. They therefore correspond to the "within animal" variation which can be established as a mean value, by a variance test procedure, in the earlier series with *Anax* and fishes. If our readings are averaged in groups of successive 3's the means of these averages are correlated, consequently we cannot by this method obtain values of  $PE$  which escape the restriction of our present method. The two human observers give  $F - \log I$  curves which are less alike than those of two individuals in our sets of *Anax* or of fishes, hence the data cannot be averaged.

The factors influencing the capacity to give variation in response (i.e., the *variability*<sup>18</sup>) can probably be kept in a more uniform state,

<sup>18</sup> Crozier, 1929



from time to time during a period of a month, in a carefully maintained set of the animals previously used than is possible with a human individual. The data show, however, that the relation of  $I_m$  to  $PE_{I_1}$  (Fig 3) is of the same kind as appears in the former cases. The curves for the 2 observers are the same, although the operators of the instrument were different. Up to about  $\log I_m = 1.5$  the two are directly proportional, and the proportionality constant (with  $PE_{I_1}$  corrected

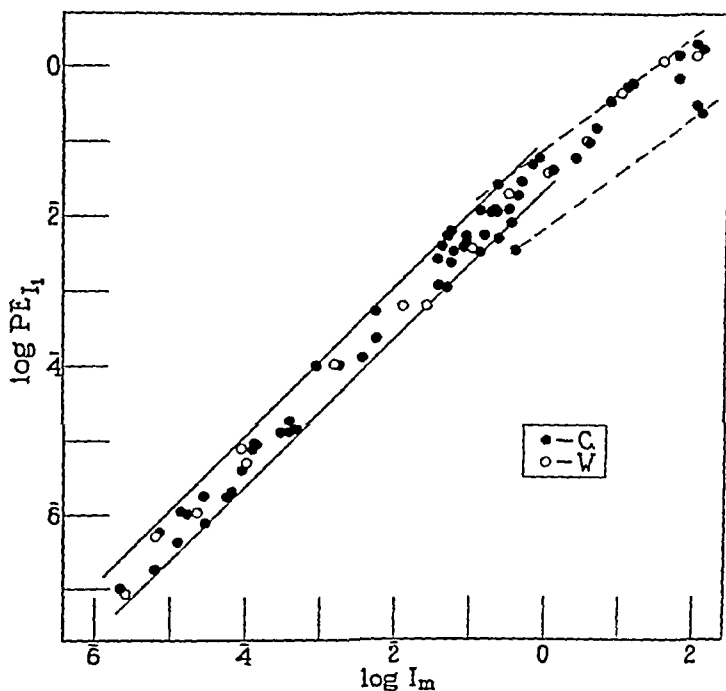


FIG 3 The scatter of  $I_1$  as a function of  $I_m$ . Solid circlets, W J C, open circlets, E W. Up to  $\log \text{intensity} = ca\ 1.5$ ,  $I_m$  and  $PE_{I_1}$  are directly proportional (slope on log grid = 1), see text

for numbers) is about the same as with other organisms. At still higher values of  $F$  the slope on a log grid becomes less. The data on fishes show the same thing<sup>16</sup>. The human  $F-I$  curve cannot be pursued to higher intensities with our apparatus, but presumably it would then show a continuation of this effect. For the two observers the course of the function is essentially the same. The lower portion, with slope = 1, gives  $PE_{I_1} = ca\ 5.4$  per cent of  $I_m$ , about one-half

<sup>16</sup> Cf also a subsequent paper on *Triturus*

the value obtained with human visual intensive discrimination<sup>17</sup> Where the slope becomes  $< 1$  (about 0.76) the proportionality can be made direct by assuming a new origin ( $\pm e$ , adding a constant to  $I_m$ , cf Crozier, 1935-36, Crozier, Wolf, and Zerrahn Wolf, 1936-37d) The change in the curve (Fig. 3) shows that  $\sigma_{I_1}$ , rather than  $\sigma_{\log I_1}$ , is the significant measure. The vertical breadth of the inclusive band is about  $1.6 \times P E_{1r}$ , which when corrected for number of observations gives 5.06—a number larger than that obtained with fishes.<sup>18</sup> The vertical breadth of the band, a little  $> 0.6$  log unit, is about the same as for human visual intensive discrimination<sup>17</sup> and less than that found with auditory and tension excitation. (These particular comparisons are merely intended to be suggestive; the interesting problems concerning the experimentally determinable properties of  $P E_I$  and of  $P E_{PE_I}$  require data of greater homogeneity before it can be decided if the proportionality between the two really has more than a simple and necessary statistical basis, the present indication is that it has.)

The variation in  $F_1$  is a slightly more complicated matter. The between individual variation, with *Anax* and with sunfish, rises to a maximum at each inflection of the  $F - \log I$  curve. The mean within individual variation also passes through a maximum, but at a definitely higher intensity. Beyond  $F_m =$  about 70 per cent of  $F_{max}$ , in each case,<sup>19</sup> the within individual variation exceeds that between individuals. This curious result is also apparent in the data of geotrophic orientation by rats (Crozier and Pincus, 1939), which are in several senses comparable to the data of visual flicker, its real significance is thus made more probable. The present measurements of  $P E_{1r}$  correspond to the indices of within animal variation, and are on the whole lower than with the other organisms. But they are complicated by the fact that in successive sets of readings a given individual does not retain the degree of uniformity in variability which is assured in the experiments with *Anax* and sunfish. The result is that the scatter of  $P E_{1r}$ , like that for  $P E_{1i}$ , is absolutely and relatively larger in these human measurements. The comparison

<sup>17</sup> Cf Crozier and Holway, 1937, Holway and Crozier, 1937 a, b

<sup>18</sup> Crozier, Wolf, and Zerrahn Wolf, 1936-37 d, 1937-38 a

<sup>19</sup> Crozier, Wolf, and Zerrahn Wolf, 1936-37 c, d

can be made by considering the behavior of the extracted mean variation, in terms of  $\sigma_r'$ , within animals, as calculated for the data<sup>1</sup> on sunfish. Each such value should carry with it a  $\sigma_\sigma$  proportional to its own magnitude (although it is possible that the proportionality factor is a function of the level of light intensity), so that on a log  $\sigma$  scale the vertical scatter of  $\sigma_r'$  should tend to be constant. Fig 4 shows that the index of variation rises to a flat maximum in the neighborhood of  $\log I = 5.5$ , and again at about  $\log I = 1.0$ , and that at still higher intensities it again increases. This agrees with the indications given

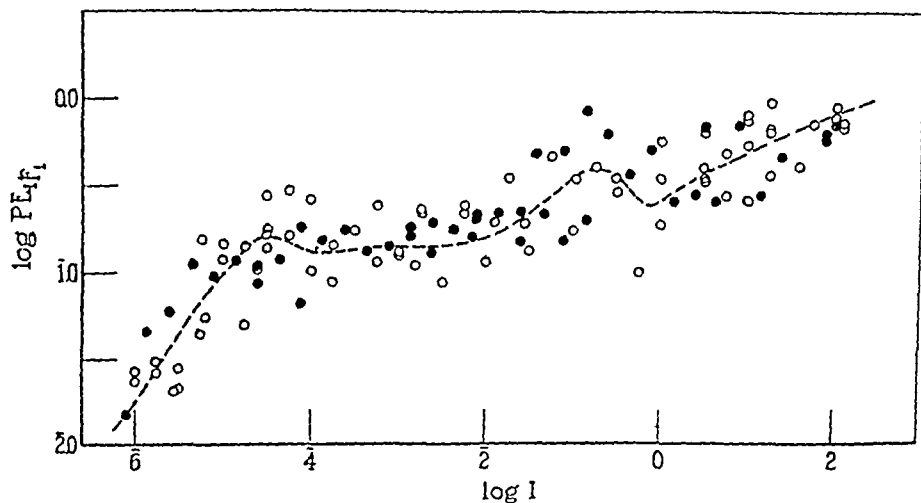


FIG 4  $P E_{I F_1}$  as a function of  $\log I$ . Solid circlets, W J C, open, E W. To facilitate comparison, the plot for C has been moved 0.1 unit to the right and 0.25 units vertically on the log grid. The vertical width of the band should tend to be statistically constant, if the same shift is appropriate for the two parts of the curves. See text.

by  $\sigma_r'$  in the sunfish data, except that the terminal increase is greater. The final increase corresponds with the increasing introduction of purely experimental errors (*cf* Crozier, Wolf, and Zerrahn-Wolf, 1936-37*a, b*) when the highest values of  $F$  are involved, it also depends upon increasing difficulty in the judgment of a consistent end-point, particularly with this apparatus in which slight lack of verticality in the cylinder holder tends to produce "ghost flickers." The fact that  $P E_r$  tends to increase, at the highest intensities, while the relative increase in  $P E_r$  becomes less, indicates, in view of the otherwise close

interconnection of the two, that purely instrumental errors there play an increasingly larger part. At the high end of each curve there is found a greater scatter of the points (Fig 2), absolutely and relatively, although  $PE_r$  increases relatively less rapidly, this indicates greater variation in the nature of the end point actually used, but consistency in its use within each set of readings.

2 The conception that, in the absence of too large a proportion of instrumental errors, the values of  $PE_r$  and  $PE_i$  are intimately related and are produced as a consequence of the basic variability of the organism<sup>1</sup> gives rise to the prediction that the curves for  $F_m$  vs  $I$  and for  $I_m$  vs  $F$  will not be identical. If many measurements are made with one individual over a period of time, the two sets of determinations should however intermingle. A test of the prediction with one animal can really be made, therefore, only by comparing pairs of determinations of  $F_m$  and  $I_m$  made in succession. When single determinations of  $F$  and  $I$  are made by a process of careful adjustment by continued small changes until a satisfactory point has been achieved, the measurements then have, of course, the significance of internally averaged  $F_m$  and  $I_m$  (Crozier, 1936), and the differences between them become more immediately obvious. The expectation<sup>1</sup> is that if  $I_m$  is found at a given  $F$ , and then  $F_m$  is obtained at this value of  $I_m$ ,  $F_m$  will be larger than  $F$  and  $F_m - I$  will pass through a maximum at each region of inflection of the  $F - \log I$  curve.

The pairs of determinations which are available for this test with our human data are plotted in Fig 5. They show that the expectation is not contradicted by the present facts. Consecutive values of  $I_m$ ,  $F_m$  show the expected differences, which separately measured pairs do not. Since the adaptation of the eye might be in a steadier state when  $F_m$  is being measured, by comparison with the condition when successive values of  $I_1$  are taken, it is clear that the data have really not been influenced by differences in the mean level of adaptation.

It is therefore possible to state that the properties of  $PE_r$  and of  $PE_i$  are consistent with those observed with lower animals.

3 The nature of the variation of  $I_1$  and of  $F_1$  is such as to suggest rather definitely that it is the whole  $F - \log I$  function which is randomly fluctuating. Whether its several parameters vary with time independently cannot be decided. The whole curve cannot be deter-

mined "instantaneously" But it is clear that in the low intensity part of the curve this kind of fluctuation is relatively more pronounced Other sets of our measurements (on W J C) show that data secured at a different time may follow a curve, in the rod region, in part separated by  $+0.2$  log unit from that shown in Fig 2 and rising to a slightly higher maximum Fluctuations of this sort have been obtained by other observers (*cf* Hecht and Smith, 1935-36) It is probable that at least part of this effect is due to fluctuation in the way in which the lower tail of the cone function is able to play a part in the response to flicker In one of the teleost species we have

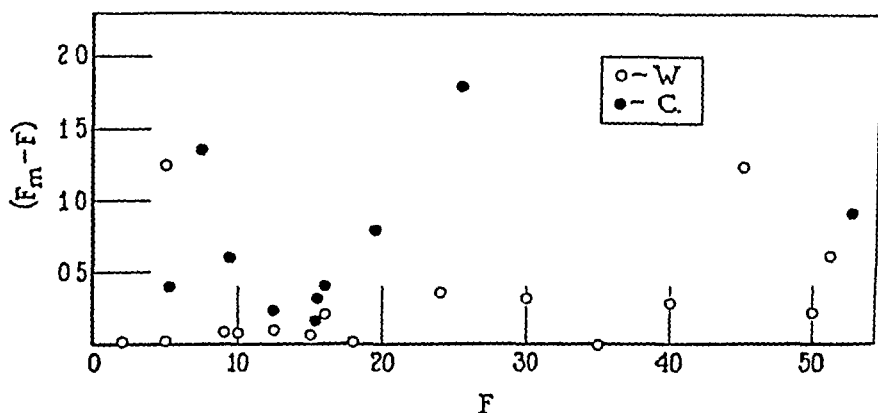


FIG 5 The difference ( $F_m - F$ ) between  $F$  and  $F_m$  at the value found for  $I_m$  at  $F$ , in series of this sort made in immediate succession, is positive Only in the regions of inflection of the  $F - \log I$  curve does it rise above 1.0 Its scatter should be greatest here also, and (for other reasons see text) at the high end of the curve

examined (Crozier, Wolf, and Zerrahn-Wolf, 1937-38a) an analogous effect was noticed, in the human curve the degree of overlapping of the rod and cone contributions is more extensive (Fig 8), and the fluctuation in the rod part of the curve would be expected to be more pronounced

It is consistent with this interpretation that considerable variation in  $I_m$  should be encountered at the intensity level deduced for the fading out of the influence of the rods upon  $F_c$ . This is at about  $\log I = 2.8$  The data in Fig 2, and other measurements, confirm this expectation It is probably significant that (Fig 3), as in our experience with fishes (Crozier, Wolf, and Zerrahn-Wolf, 1936-37d, 1937-

38a), the "break" in the curve of  $\log P E_{r_1}$  vs  $\log I_m$  comes just beyond the point at which the declining rod curve stops

## IV

The flicker response curves ( $F$ ,  $\log I$ ) for fishes have been resolved into two additively component probability integrals, respectively the contributions made by rods and by cones to the determination of  $F$ . The structure of the curves shows that beyond a certain intensity the effectiveness of the rods declines. The diminishing contribution

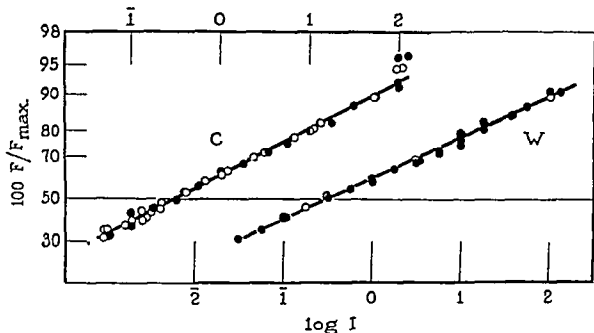


FIG 6  $F$  vs  $\log I$  on a probability grid. Solid circle,  $F_m$ ; open circle,  $I_m$ , the data of Fig 2, above  $F = 18$ . For C  $F_{max} = 56.0$ , for W, 59.0 (The limits of horizontal scatter of the points agree with calculation on the basis of Fig 3. The extreme departures at the upper end of the C curve (3.8 per cent) are commented on in the text.)

of rod function follows a reversed probability integral.<sup>1</sup> Arthropods with large convex eyes give asymmetrical  $F$ ,  $\log I$  curves, the distorted probability integral form of these curves has been shown to be due to the mechanically disadvantageous location of the marginal ommatidia of the eye, reducing their contribution at lower intensities.<sup>20</sup> The data on human flicker fusion, in the cone part of the curve, are well described<sup>4</sup> by a probability integral. The separation of the centers of the rod and the cone sections of the human flicker curves is relatively less on the  $\log I$  axis than with the fishes, and the total rod

<sup>20</sup> Crozier, Wolf, and Zerrahn Wolf, 1937-38, b, c.

effect is larger. This brings it about that the overlapping of the zones of activity of rods and cones in human flicker recognition is more extensive, although with the fishes the decay curve for the rods is at a relatively higher intensity and more widely separated from the ascending rod curve.

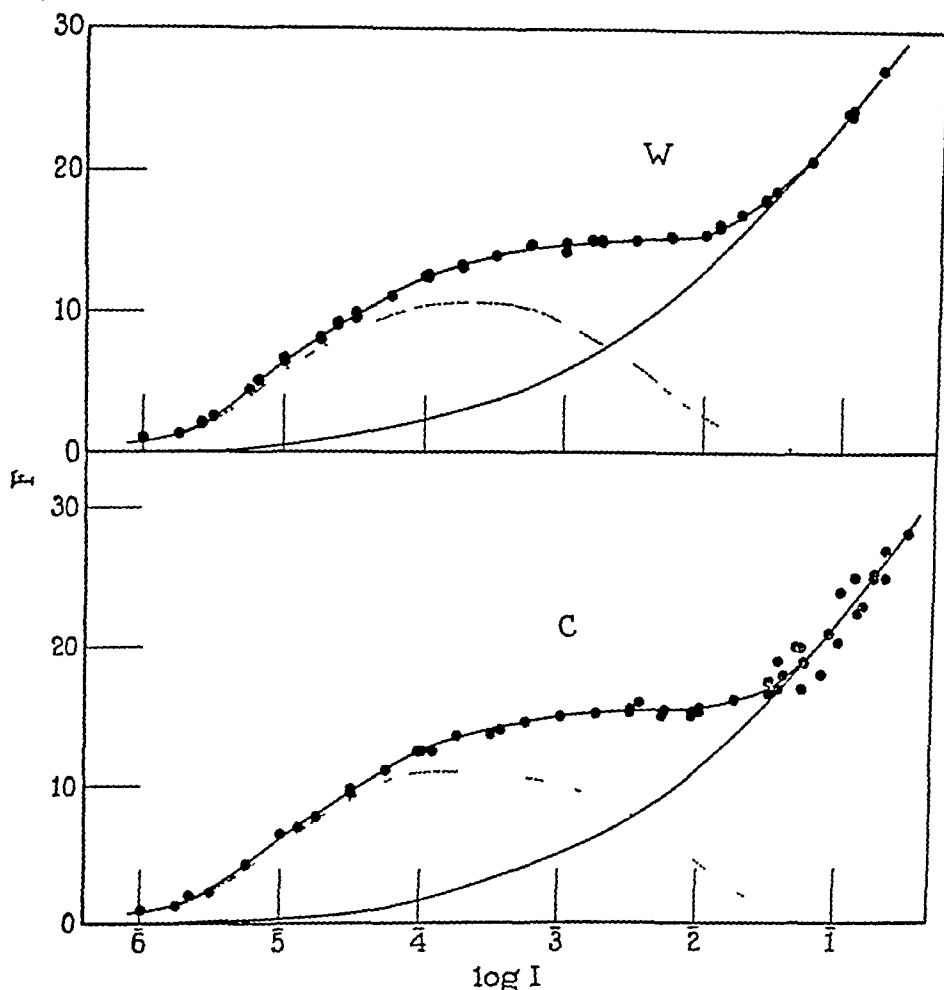


FIG 7 The lower parts of the curves of Fig 2. The probability integrals of Fig 6, for the cone portions, have been extrapolated to  $F = 0$ . The difference curve is shown, dotted, its rising and falling branches are each fitted by a probability integral (Fig 8).

The curves in Figs 6, 7, and 8 show that this analysis gives an adequate description of the human flicker response measurements. The maxima  $F_{max}$  are found by trial; the experimental difficulties in the production of higher intensities, the effects of glare, and other

conditions make it impossible to complete the curves with this apparatus. There is very small latitude indeed, however, in the selection of  $F_{max}$  for a reasonably fitting curve. The test of a good fit is (1) that it obviously runs through the plotted points, (2) that it contributes adequately to the resolution of the composite graph, (3) that its parameters have the properties rationally expected of the standard deviation, maximum, and abscissa of inflection of a probability integral of excitation elements. The experiments with different kinds of organisms<sup>4, 7</sup> show that these criteria have been met fairly well.

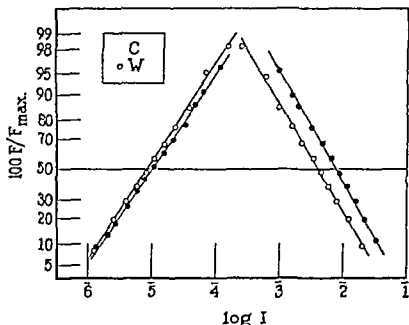


FIG 8 The rising and descending portions of the rod contribution curve obtained in Fig 7 are each described by a probability integral. See Table I. (The points are read from the curves in Fig 7.)

The cone parts are well fitted, down to  $ca F = 18$  (Fig 6). The extension of the probability curve to the  $\log I$  axis gives by difference the (dotted) curve attributed to rod activity in the discrimination of flicker (Fig 7). The ascending and the descending limbs of these curves are also well fitted by probability integrals (Fig 8). In our measurements with fishes the separations of rod and cone components of the curves are so great that the entrance of cone activity produces a distinct bump on the flat intermediate part of the graph. This "anchoring point" for the extrapolation of the cone integral is not available in the human data. It has been pointed out<sup>21</sup> that in different organisms cases are to be expected in which the extent of over

<sup>21</sup> Crozier, Wolf, and Zerrahn Wolf, 1936-37 *d* p 430



lapping of rod and cone curves varies. When overlapping is so complete as in Fig 7, a test of the resolution by extrapolation of the cone curve can be made by seeing if the *uncorrected* rod data follow a probability integral. Tests prove that they cannot be rectified on a probability grid.

The curves for our two observers show the same kind of structure despite differences in the parameters of the rod and cone functions (Table I). The cone maximum and rod maximum differ independently (as proved, also, in our experiments with fishes<sup>18</sup>). But a higher maximum goes with a higher intensity ( $\tau'$ ) at the inflection point and larger proportionate spread of the underlying frequency distribution of effects as a function of  $\log I$ . This is also indicated by the data of other observers<sup>10</sup>. There is indicated throughout these comparisons a

TABLE I

*Constants for the Flicker Response Curves of Figs 2 and 8,  $F$  As Percentage of  $F_{max}$  vs  $\log I$ .  $\tau' = \log I$  at Inflection of the Curve*

Parameter	W J C	E W
$F_{max}$ , cones	56.0	59.0
$F_{max}$ , rods	11.6	10.5
$\tau'$ , cones	1.48	1.50
$\tau'$ , rods	5.00	6.93
$\tau'$ , declining rod curve	3.87	3.61
$\sigma \log I$ , cones	1.14	1.28
$\sigma \log I$ , rods	0.42	0.40
$\sigma \log I$ , declining rod curve	0.34	0.35

consistency with the expectations derived from the conception that the flicker response curve ( $F$ ,  $\log I$ ) is essentially a probability integral of which the parameters are determined by the structural organization of the particular individual<sup>22</sup>. The properties of these parameters as functions of genetic composition, age, experimental treatments, and other factors, present numerous significant problems.

## V

## SUMMARY

Using the rotating striped cylinder device previously employed for determination of the flicker response function with lower animals,

<sup>22</sup> Crozier, Wolf, and Zerrahn-Wolf, 1937, 1937-38 a

corresponding measurements have been made with human observers. The curves based upon the relation between critical flash frequency and critical intensity for the signalling of the recognition of flicker have the properties of human flicker fusion data as obtained by other methods. They also have the quantitative properties of the flicker curves provided by the motor responses of insects and fishes to the seen movement of flashes. This applies to the variation found in repeated measurements as well as to the nature of the analytical function describing the connection between flash frequency and intensity. The data for human visual flicker and those for the responses of lower animals are therefore essentially homologous.

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# CRITICAL ILLUMINATION FOR RESPONSE TO FLICKERED LIGHT, WITH DRAGONFLY LARVAE (ANAX), IN RELATION TO AREA OF EYE

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## PLATE 1

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### I

Data on reaction to visual flicker have been obtained with various animals. Mean values of critical illumination ( $I_m$ ) for response are secured at different frequencies of flashing ( $F$ ), a fixed proportion of the flicker cycle being occupied by light, or, mean values of  $F_m$  as a function of illumination  $I$ . The curves for  $I_m$  vs  $F$  and for  $F_m$  vs  $I$  are not identical when measurements are made with the same group of organisms (Crozier, 1935-36, Crozier, Wolf, and Zerrahn Wolf, 1936-37 *a*, *b*). Such data give a starting point for the theory of excitation by intermittent illumination, since it is only the energy level for threshold response to flicker (or for marginal suppression of flicker, in some cases) which can really be measured directly and objectively. The technique of the experiments is applicable for a number of different kinds of animals,<sup>1</sup> however, and for a diversity of tests to which the theory of response to intermittent light should be subjected. Among the variables to be examined are temperature,<sup>2</sup> area of excitable surface, and the proportion of light time ( $t_L$ ) to dark time ( $t_D$ ) in a flicker cycle.<sup>3</sup> The properties of the  $F - I$  curve as related to these variables supply criteria which the theory of excitation by flicker must satisfy.

<sup>1</sup> Cf. Wolf 1933-34, Wolf and Zerrahn Wolf, 1935-36, Crozier, Wolf, and Zerrahn Wolf, 1936-37 *a*, *b*, 1937-38 *a*, and other forms not as yet systematically examined.

<sup>2</sup> Crozier, Wolf, and Zerrahn Wolf 1936-37 *c*, *d*.

<sup>3</sup> Crozier, Wolf, and Zerrahn Wolf 1937-38 *b*, *c*.

The view<sup>4</sup> we have adopted for testing is that response to flicker depends upon sensory discrimination between the effects of flashes of light and the after effects in the intervals of no light. A mean critical intensity  $I_m$  has thus the dimensional properties of  $\Delta I_m$  in an intensity discrimination measurement so arranged that  $\tilde{I}_2 - \tilde{I}_1 = \tilde{I}_2 - 0 = \Delta I$ <sup>4</sup>. One important such property is quite apparent experimentally<sup>5</sup> since  $d I_m = k d \sigma_{1I}$ , this being the relationship uniformly encountered for the *minimal discriminable intensity interval*  $\Delta I$ <sup>6</sup>. The mechanism of intensive discrimination leads to the expectation that for the case of marginal discrimination of flicker a lowering of the temperature must lead to an increase of  $I_m$  at given  $F$ , or lower  $F_m$  at fixed  $I$ , which is found<sup>3</sup>. It is also to be expected that as  $t_L/t_D$  is altered by increasing it from low values to high, the  $F - \log I$  curve must move toward higher intensities, become in a general way less steep, and attain a lower maximum value of  $F$ <sup>7</sup>. If at fixed intensities the area of the visual receptor surface is decreased, the curve should be shifted toward lower values of  $F$ <sup>8</sup>. Disregarding certain differences (concerning the behavior of  $F_{max}$ ), the effects of increasing  $t_L/t_D$ , of decreasing retinal area, and of lowering the temperature should thus all produce the same general kind of change in the  $F - \log I$  curve. It is to be noted that from the standpoint of the simple theory that such data refer to the properties of the primary excitation process in the retina<sup>9</sup> these predictions, experimentally verified, are not mutually consistent, certain of them are definitely inconsistent with the present form of the photochemical interpretation of the data on response to flicker,<sup>2</sup> their net result is to indicate that the data of visual response depend immediately upon properties of the organism

<sup>4</sup> Crozier, 1935-36, 1936, Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *c, d*.  $\tilde{I}$  signifies a mean value of an adjusted critical intensity, *cf* Crozier and Holway, 1937

<sup>5</sup> Crozier, 1935-36, 1936, Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *b, c, d*, etc

<sup>6</sup> Crozier, 1935-36, Crozier and Holway, 1937

<sup>7</sup> Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b, c*. The analysis of the data in the literature of human reactions to flicker is complicated by the fact that two different procedures have been adopted in tests of the effect of modifying the ratio  $t_L/t_D$ , which necessarily give two contrasting results since the nature of the test is radically different in the two cases. This is discussed in following papers

<sup>8</sup> *Cf* Hecht, Shlaer, and Smith, 1935, Hecht and Smith, 1935-36

<sup>9</sup> *Cf* Hecht, 1931, 1937

as a reacting system, rather than upon the nature of the initial events of photoreception<sup>10</sup>

It has been proposed to consider that the critical excitation at any level of  $F$  is the accumulated result of the action of a large number of "elements of excitation" Such an element is defined in units proportional to  $dF/d \log I_m$ <sup>11</sup> It is not supposed that a given retinal unit of structure corresponds to one element, each receptor unit fluctuates in its capacity to contribute to the determination of the index response The origination of log frequency distributions of element thresholds is thus not difficult to understand Excitation up to the level of forced reaction to flicker will then be conceived to result from the summation of excitation units of this sort, and will be proportional to  $F$  A quantitative substantiation of this conception, in these terms, is provided by the fact that the integral of  $\sigma_r$  vs  $\log I$  has the properties of  $F$  and for each value of  $\log I$  is directly proportional to  $F$ <sup>12</sup>

The curve of  $F - \log I$  is therefore regarded as having the nature of a population curve—approximately a logistic, or better a probability integral<sup>13</sup> Such a curve describes the data in simple cases The standard deviation of the underlying frequency distribution is independent of temperature<sup>14</sup> and of certain other variables, and in breeding experiments involving an interspecific cross it gives further evidence, of another kind, that it possesses the qualities of a biologically significant invariant,<sup>14</sup> determined by the structural organization of the animal considered An integral of this sort describing the magnitude of the total excitation accumulated from a population of excitable elements at the point of threshold reaction, as a function of intensity, involves no assumptions whatever as to the intimate mechanism of excitation in any single element or in the available population of retinal units, further, it is not assumed that the elements of excitation,

<sup>10</sup> The properties of  $\Delta I$ , and thus<sup>4</sup> by inference the properties of  $I$  for response to flicker are independent of the nature of the peripheral sensory field Crozier and Holway, 1937 Upton and Crozier, 1936

<sup>11</sup> Crozier, 1935–36 1936, 1937 and footnotes 2 and 3

<sup>12</sup> Crozier, Wolf and Zerrahn Wolf, 1937–38 *a*

<sup>13</sup> Crozier, Wolf, and Zerrahn Wolf, 1936–37 *b, c, d* 1937–38 *a b c* Crozier, 1937

<sup>14</sup> Crozier, 1937, Crozier, Wolf and Zerrahn Wolf, 1937–38 *a c*

defined as a function of  $I$ , are even primarily an expression of peripheral receptor properties at all

The eyes of certain arthropods present no obvious evidence that they contain the kind of structural complexity of the retina found in typical vertebrates. The curves which they provide for excitation as a function of  $\log I$  do not, however, follow the law of a simple logistic or probability integral<sup>15</sup>. The discrepancy (with  $t_L/t_D = 1$ ) is in the nature of a systematic deficiency in  $F$  over lower values of  $I$  (*Anax junius*, *Apis*). It was suggested<sup>15</sup> that at lower intensities certain ommatidia which might provide elements of excitation in the sense in which we have defined them are at a mechanical disadvantage in the reception of light. Perhaps because certain ommatidia are at too great an angle to the light, they do not receive sufficient energy to enable them to take part in the process whereby flicker is reacted to under the condition that a sufficient magnitude of excitation for this purpose is obtained from other units of the retina. At much higher intensities (with higher values of  $F$ ) the chance of these disadvantaged units being successfully implicated should be greater, and their effect accordingly perceptible. Somewhat similar considerations apply to the interpretation of tests of visual acuity<sup>15</sup>. Consequently, a suitable experiment might show that blocking out certain regions of the eyes should obliterate, or tend to obliterate, the departure of the  $F - \log I_m$  curve from the expected integral form (with  $t_L/t_D = 1$ ). A similar result would be expected from an increase of  $t_L/t_D$  in each flicker cycle<sup>16</sup>. Of course, the possibility exists that certain units in any or all parts of the retina may be inactive at low intensities. Dolley and Wierda (1929) concluded that the central area of the retina of *Eristalis* is much more effective in phototropic excitation, according to Dolley and Hartwell (1936) the photic sensitivity of the ommatidia, as expressed by induced turning responses, is maximum at about the center of the eye, and is minimum at the posterior edge (cf. also Ludke, 1935).

The chief purpose of the present experiments was to examine the nature of the asymmetry of the *Anax* flicker curve, by means of tests in which different parts of the eye have been covered, rather than to establish the relations between  $F$ ,  $I$ , and area. Human visual flicker

<sup>15</sup> Crozier, Wolf, and Zerrahn-Wolf, 1936-37 b, c

<sup>16</sup> Cf. Crozier, Wolf, and Zerrahn-Wolf, 1937-38 b

data show<sup>8</sup> that at fixed  $F$  an increase in area of retina exposed to stimulation results in a decrease of  $I_m$ . This corresponds to the fact that for visual<sup>17</sup> intensity discrimination and for intensive discrimination in auditory<sup>18</sup> and pressure<sup>19</sup> excitation,  $\Delta I$  at fixed  $I_1$  is an inverse function of sensory area exposed (in the flicker case,  $I_1 = 0$  and  $I_m \equiv \Delta I$ ). With the human data, the fundamental form of the  $F - I_m$  function is not much if at all dependent upon area,<sup>8</sup> but with *Anax* it is markedly affected.

Our interpretation of threshold or marginal response to flicker has been in terms of a kind of intensity discrimination.<sup>4</sup> The theory of this discrimination calls for a proportionality between  $\Delta I_m$  and  $\sigma_{\Delta I}$  which (for a given sensory field) is independent of the extent of area excited and of certain other variables.<sup>6</sup> If this conception of marginal recognition of flicker is adequate,  $I_m$  should be the same function of  $\sigma_I$  without respect to area of retina. It should also be independent of the imputed distortion of the *Anax*  $F - I$  curve, provided only a single class of receptor units is concerned in the excitation responsible for the index reaction. With a typical vertebrate, the  $I - \sigma_I$  curve is broken into two portions, as is that for  $F$  vs  $I$  (although not in exactly the same way), for visual intensity discrimination data<sup>6</sup> it is not, since in this case two real intensities ( $I_1$  and  $I_2$ ) are being compared. With area as variant, the constancy of the relation between  $I_m$  and  $\sigma_I$  is in the same status as that between  $\Delta I_m$  and  $\sigma_{\Delta I}$ ,<sup>6</sup> the homology of  $I_m$  and  $\Delta I_m$  is therefore emphasized.

## II

The plan of the experiments was to obtain  $F - \log I_m$  curves for groups of ten *Anax* nymphs in which various portions of the eye surface had been covered by an opaque enamel. It turned out that small differences in the surface covered in different individuals designedly prepared in the same manner could be shown to be without significance. Thus, as is indicated subsequently (Text fig. 3) the variation of  $I_1$  for given  $I_m$  is the same in various groups with partially covered eyes as in the normal animals with eyes not painted. Probably this is in part due to the fact that small differences in number of ommatidia covered are really not significant. In part it is also due to the fact that light leakages occur through and around the edge of the opaque covering.

<sup>17</sup> Cf. Abney and Watson, 1916 Steinhardt 1936-37

<sup>18</sup> Crozier and Holway 1937 Holway and Crozier 1938

<sup>19</sup> Wolf, 1932-33, Hecht and Wolf 1928-29



For blackening desired areas of the *Anax* eyes there was used, after trial of various things, a mixture of "Kodalak" with a small amount of a saturated solution of white shellac in absolute ethyl alcohol. The addition of the shellac in alcohol causes the paint to adhere better and to dry faster, it must not run too freely. It was applied with a small camel's hair brush. The larvae were taken out of water, and the posterior part of the body, to the anterior legs, was wrapped in moist cheese cloth. The eyes were thoroughly dried with absorbent cotton. Painting was done under the binocular microscope, 12.5 magnification, with illumination by a microscope lamp. Both eyes of a wrapped-up larva can be painted at one time. This makes it easier to prepare the two eyes in approximately the same way. If more than one patch is to be applied at one time on one eye, the first must dry before the second is applied (this prevents spreading).

After painting the larvae were put for at least one-half or three-quarters of an hour in glass jars with a piece of slightly moistened paper towel. When the paint was dry they were returned to water. It frequently happens that the edges of the painted areas crack off. Therefore, each individual was examined every day before measurements were undertaken, and breaks in the opaque eye-caps were repaired.

The lacquer does not injure the *Anax* eye. The opaque coverings can be broken off after several weeks, and the larvae then give normal flicker responses. The covered regions at first show the retinal pigment in the "dark" position upon uncovering, but it quickly migrates in the light to the light adapted location.

Measurements were made at two flash frequencies on each day. The procedure was that already described in our earlier papers.<sup>1, 2</sup> Three measurements were made of  $I_c$ , the critical intensity of illumination, with each of the ten larvae, the average of these three values of  $I_c = I_1$ . The mean value of  $I_1$  was recorded as  $I_m$ ,  $P.E.I_1$  was computed from the averages for the ten larvae in a given group. This gives an estimate of the interindividual variation on a basis which is intended to reduce instrumental and other extraneous observational errors. This is the method we have followed in previous papers.<sup>1, 2</sup> In the present data, however, the variation of  $I_c$  within individuals is practically the same over the whole range of intensities as the variation between individuals. There is little doubt that this is due to the fact that in successive tests the orientation of a larva with respect to the revolving cylinder<sup>1</sup> is not identical, so that there arises the possibility of a variable light leakage around and through the edge of the opaque eye-cap. This situation is not involved when temperature<sup>3</sup> or relative length of light interval<sup>7</sup> is varied, and in those cases the interindividual variation bulks larger.

A new and steadier magneto and a very much more sensitive voltmeter for the regulation of  $F$  were substituted in our apparatus.<sup>1</sup> This had no detectable effect upon the readings of  $I_c$ . In some ten series of measurements, however, made for various purposes,  $\sigma_{I_1}$  is slightly but consistently lower at given  $I_m$  (or,  $I_m$  is higher for given  $\sigma_{I_1}$ ) as compared with a series made a year or more previously at the same temperature.<sup>14</sup> The "spread coefficient" of  $\sigma_{I_1}$ ,<sup>3</sup> however, is identical with that observed previously. This, together with the fact that

no progressive change in  $\sigma_{I_1}$  is observable during a prolonged series of observations, makes it impossible to assume that the slight change in the  $\log \sigma_{I_1}$  vs  $\log I_m$  plot is due to the observers. It is more properly attributed to the use of animals from a different source. It is shown in the data for normal larvae from these collections as well as for those with partially covered eyes.

At various times additional determinations of  $I_m$  were made for normal larvae at different levels of  $F$ , while the tests with partly covered eyes were in progress. They agreed in a very satisfactory way with the values already obtained<sup>14</sup> at  $21^\circ$  for  $t_L/t_D = 1$  (Table I).

Five groups of larvae were prepared in which different parts of the eye surface had been covered. The preliminary expectation was that a central region of the ommatidial surface should be found most nearly to approach the ideal condition,—in other words, the  $F - I$  curve from this region might most closely approach a symmetrical probability integral. (1) The posterior half of the eye was covered, (2) the anterior half was covered, (3) a median vertical stripe on the eye was left uncovered, (4) a median vertical stripe was painted over, (5) a circular patch on the posterior central portion of the eye surface was left free.

The behavior of the *Anax* nymphs prepared in these ways differed from that of normal larvae as regards their orientation with reference to the rotating striped cylinder, and also in their movements constituting the threshold reactions to flicker. This could be responsible for an impression of regional differences in 'sensitivity' over the surface of the eye. For example, if one exposes to flickering light having a flash frequency of 40/sec, (a) larvae with the posterior half of the eyes covered, response is obtained at  $\log I_m = 1.43$ , (b) the anterior half of the eyes covered, at  $\log I_m = 1.22$ , (c) the vertical central stripe of the eyes covered reaction is apparent at  $\log I_m = 1.20$ . In terms of the maximum flicker frequency for each type of preparation  $\log I_m$  at  $100 F/F_{mo} = 76$  is for (a) 1.42, (b) 1.25, (c) 1.25. The magnitudes of these differences, however, depend upon the level of  $F$  (cf. Text fig. 1), and without further information would need to be interpreted in terms of the reactions exhibited by the several types of prepared larvae. It should be added however, that measurements based upon the use of the 'catching' reaction of the labium give data, with normal larvae, which do not differ significantly from those based upon forced swimming movements.

(1) *Posterior Half of Eyes Covered*—Larvae face the striped cylinder. Reaction at the critical illumination is not so sharp as with the whole eye functioning. At the point of response, raising and lowering movements occur due to contraction of leg flexors producing some bumping against the wall of the container then swimming movements are begun, mostly in the direction of the motion of the stripes but often in the opposite direction.

(2) *Anterior Half of Eyes Covered*—To obtain a response the larvae must be oriented with their long axes parallel to the wall of the container. Swimming occurs in the direction of motion of the stripes. The response is clear and sharp as with normal animals.

(3) *Vertical Central Section of Eyes Uncovered*—Larvae must be nearly or quite

parallel to the wall. Response is in the form of swimming with the motion of the stripes. Onset of reaction is not so sharp as with the posterior half of the eye functioning (2), but is sufficiently reliable as an index.

(4) *Vertical Central Section of Eyes Covered*—If the animals directly face the striped cylinder, the response to flicker is as in (1), and the anterior ends bump the wall of the container. If the larvae then turn, it is usually in the direction of the stripe motion. When the animals are parallel to the cylinder wall, response is as with (2), and is precise.

(5) *Central Area of Eyes Surface Open*—The behavior is quite like that described under (3), naturally enough, but, since the exposed area is less the reactions are less abrupt.

Photographs of partially covered *Anax* eyes of the five types used are given in Figs 1-5.

### III

The data obtained with *Anax* nymphs having different portions of the eyes opaquely covered are set out in Table I. Before and after these experiments, determinations were also made of  $I_m$  at various flash frequencies with normal larvae. These showed excellent agreement with the corresponding values of  $I_m$  previously obtained<sup>2</sup> at the same temperature (21.5°) and with the same ratio of light time to dark time in the flicker cycle ( $t_L/t_D = 1$ ). Repetitions of determinations of  $I_m$  for sets of *Anax* with the eyes partially covered in a particular way (cf Table I) do not give so close an agreement. Since (a) different eyes cannot be prepared in exactly the same manner, and since (b) variations must arise from differences in the successive initial orientations of an individual with respect to the striped cylinder, affecting leakage of light through the cap of enamel and around its edge, no such closeness of agreement could be expected.

The rôle of the sources of variation in  $I_1$  just mentioned is clearly shown by the fact that whereas with normal larvae, except at high illuminations, the within-animal variation of the critical intensity  $I_c$  is less than that between animals,<sup>2</sup> in the covered eye series this is not the case, here, the within-animal and the between-animal variations are the same, a fact which gives significant evidence for the reality of the meaning of the values of  $P E_{1r} (= k \sigma_{1r})$  as we have computed them in our standard procedure with normal larvae. This property of the measurements is again exhibited, however, in normal eye series in which  $t_L/t_D$  is varied.<sup>7</sup> The difference between the varia-

tion of  $I_e$  in sets with and without partial covering of the eyes does not affect the relationship between  $I_m$  and  $\sigma_{I_1}$  (cf Text fig 3)

To give but one example of the findings of all five series with eyes partially covered we may take set (1), in which the posterior half of the eye was painted over. Here the mean of the ten individual average rank order positions for  $I$  was 5.49 with  $PE_1 = 1.12$  the maximum departure  $= 2.66 \times PE_1$  the extreme numbers differing by  $2.40 \times PE_1$ . This is the situation encountered for fluctuation of individuals in our earlier series.<sup>3</sup> There is no correlation between rank numbers in successive tests, however. A variance test shows that the value of  $\sigma$  for the *between*-animal differences is not significantly different from that *within* the readings obtained for each individual. For normal larvae, except at the highest values of  $F$ ,<sup>3</sup> the *between*-animal variation is larger, as is also found with other organisms tested.

Thus the variation in  $I_e$  is the same from one larva to another as between readings on a given larva. The sources of variation already noted (section II) operate to obliterate the otherwise encountered individualities of the several nymphs.

The curve for  $PE_{I_1}$  vs  $I_m$  is slightly but consistently lower in the series with partial eye covering than in our earlier<sup>2</sup> normal series at this temperature. These new data, however, agree quantitatively with the values for normal animals from the same new lot (Text fig 3) and with those from experiments with the same stock in which  $t_L/t_D$  was varied.<sup>7</sup> The difference must be attributed to a primary difference in the stocks of animals. The new lot came from a different locality, but its care and treatment were the same. There is no real evidence for assuming a progressive drift with time (i.e., due to experience of the observers), similar differences between different lots of other organisms tested have also been observed.

Text fig 1 is a plot of the data in Table I. It is apparent that reduction of eye area results in a lowering of the  $F - \log I_m$  curve. There is no apparent simple relationship to extent of functioning area. In addition to increasing  $I_m$  at fixed  $F$ , the shape of the curve is also altered. This is easily seen when  $F$  is taken on a percentage scale (Text-fig 2).

The relation of  $I_m$  to  $PE_{I_1}$  is the same for all five sets of tests with partially covered eyes (Text fig 3) as for the normal larvae. This is consistent with the intensity discrimination conception of response to flicker. On this basis,<sup>5</sup>  $I_m$  corresponds to  $\tilde{I}$  in the relationship

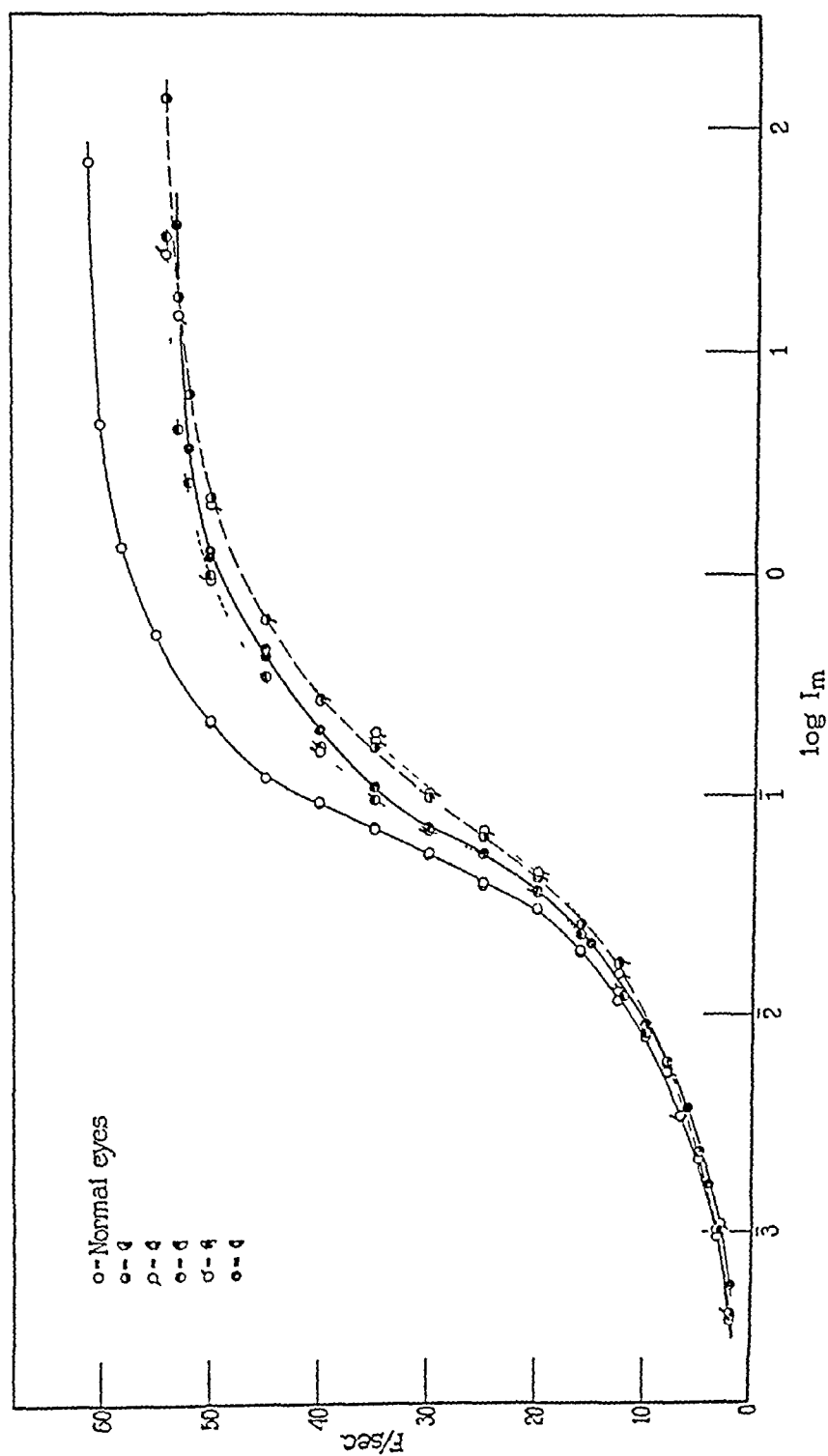
TABLE I

Mean critical intensities (*millilamberts*) for response to flicker, as a function of flicker frequency  $F$  *Anax junus* nymphs, normal and (1 - 5) with various areas of the eyes opaquet,  $P E_{I_1} = P E$  of the dispersion of thirty readings, three on each of ten larvae,  $t_L/t_D = 1$ , temperature = 21.5°

The bold-face entries under "Normal" represent determinations made with nymphs of the lot used in 1 - 5, the other figures are from Crozier, Wolf, and Zerrahn-Wolf, 1936-37b (The averages for new and old series respectively agree very closely, the  $P E$ 's for the new lot are consistently lower, as discussed in the text)

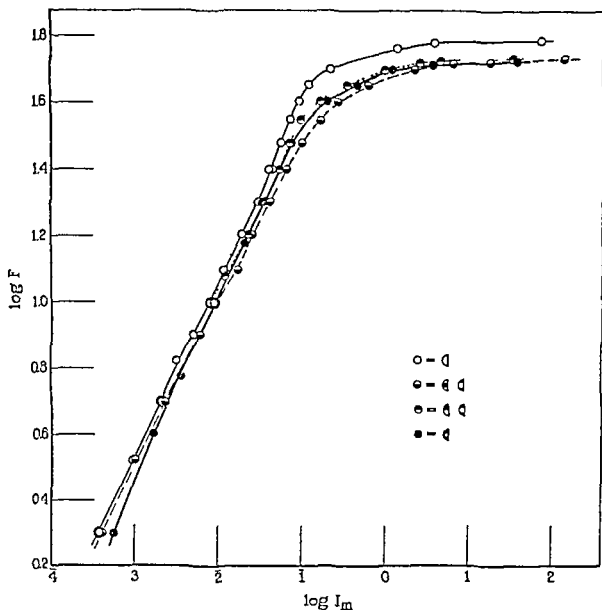
$\Gamma/\text{sec}$	Normal		(1)		(2)		(3)		(4)		(5)	
	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$
2	$\bar{4}$ 5884	$\bar{5}$ 3210	$\bar{4}$ 6201	$\bar{5}$ 2665	$\bar{4}$ 6087	$\bar{5}$ 2180	$\bar{3}$ 0394	$\bar{5}$ 7781	$\bar{4}$ 6148	$\bar{5}$ 4616	$\bar{4}$ 7510	$\bar{5}$ 4381
3	$\bar{4}$ 9784	$\bar{5}$ 7059	$\bar{3}$ 0101	$\bar{5}$ 4913								
3 33	$\bar{3}$ 3367	$\bar{4}$ 4797	$\bar{3}$ 3729	$\bar{5}$ 9490	$\bar{3}$ 3685	$\bar{5}$ 5541						
4												
5												
6												
6 66												
6 72	$\bar{3}$ 5376	$\bar{4}$ 2761									$\bar{3}$ 2398	$\bar{5}$ 5454
8	$\bar{3}$ 7308	$\bar{4}$ 6394									$\bar{3}$ 5717	$\bar{4}$ 0895
10	$\bar{3}$ 8982	$\bar{4}$ 9282	$\bar{3}$ 7807	$\bar{4}$ 3226	$\bar{3}$ 7763	$\bar{4}$ 3843	$\bar{3}$ 7395	$\bar{4}$ 4124	$\bar{3}$ 5359	$\bar{4}$ 1997		
12			$\bar{3}$ 9460	$\bar{4}$ 4669	$\bar{3}$ 9106	$\bar{4}$ 4281						
12 5	$\bar{2}$ 0649	$\bar{4}$ 6585	$\bar{2}$ 2340	$\bar{4}$ 8249	$\bar{2}$ 0842	$\bar{4}$ 7773						
15							$\bar{2}$ 1898	$\bar{4}$ 6145	$\bar{2}$ 1062	$\bar{4}$ 5464	$\bar{3}$ 9589	$\bar{4}$ 6811
16	$\bar{2}$ 2830	$\bar{3}$ 1593					$\bar{2}$ 2405	$\bar{3}$ 1981				
	$\bar{2}$ 2962	$\bar{4}$ 9197	$\bar{2}$ 4121	$\bar{4}$ 8066	$\bar{2}$ 3642	$\bar{3}$ 0281					$\bar{2}$ 3232	$\bar{4}$ 9174

20	2 4881 2 4850	3 2248 4 9845	2 6252 3 1202	2 5844 4 9420	2 6313 2 6548 2 6380	3 0306 3 5192 3 4152	2 5656 4 9499	2 5622 3 2622	3 2622
25	2 6019 2 6086 2 5958 2 5987 2 7449 2 7403 2 8592 2 8523 2 9696 2 9783	3 4682 3 4682 3 4370 3 010 3 4360 4 9216 3 4166 3 2684 3 6885 3 3766	2 8135 3 6672	2 7366 3 1572	2 8435 2 8492	3 4652 3 0338	2 7400 3 0354	2 7400	3 0354
30	2 7449 2 7403 2 8592 2 8523 2 9696 2 9783	3 4360 4 9216 3 4166 3 2684 3 6885 3 3766	2 9923 3 2164	2 8500 3 3988	1 0187	3 6368	2 8426 3 2479	2 8586 3 3465	3 3465
35	2 8592 2 8523 2 9696 2 9783	3 4166 3 2684 3 6885 3 3766	1 2196 3 7634	2 9842 3 4613	1 2982 1 2504	2 1258	1 0465 3 3284	1 0465	3 3284
40	2 9696 2 9783	3 6885 3 3766	1 4310 3 9916	1 2214 3 5699	1 4440	3 8173	1 2006 3 5833	1 3019 2 0038	2 0038
45	1 0874	3 6669	1 7993 2 1878	1 5412 2 1758	1 8052	2 3510	1 6654 1 6387	1 6654 1 6387	2 1408 3 8584
50	1 3438 1 3384	2 0245 3 9829	0 3475 2 8541	1 9991 2 1813	0 3132	1 0277	1 9723 2 4858	0 0813 0 1099	2 5145 2 2100
52			0 8107 1 3351	0 4173 1 0723	1 1608	1 7050		0 5695 1 5654	1 0314 0 3502
53			1 2485 1 7608	0 6538 1 3585					
54			2 1281 0 7881	1 5122 0 6062					
55	1 7356	2 4538					1 4320		
58	0 1252								
60	0 5759	1 2390							
61	1 8407	0 8341							



TEXT-FIG 1  $\log I_m$  as a function of flash frequency  $F$  for normal *Anax* nymphs, and for groups with parts of the eyes opaqued (Figs 1-5) as indicated. Data in Table I

$\bar{I}_2 - I_1 = \Delta I_m$ , and  $I_1$  is zero, we find<sup>6</sup> in all cases that  $P E_{\Delta I} = k \Delta I_m$  and that  $k$  is not a function of area. With regard to sharpness and definiteness of the response used as index of reaction to flicker there is



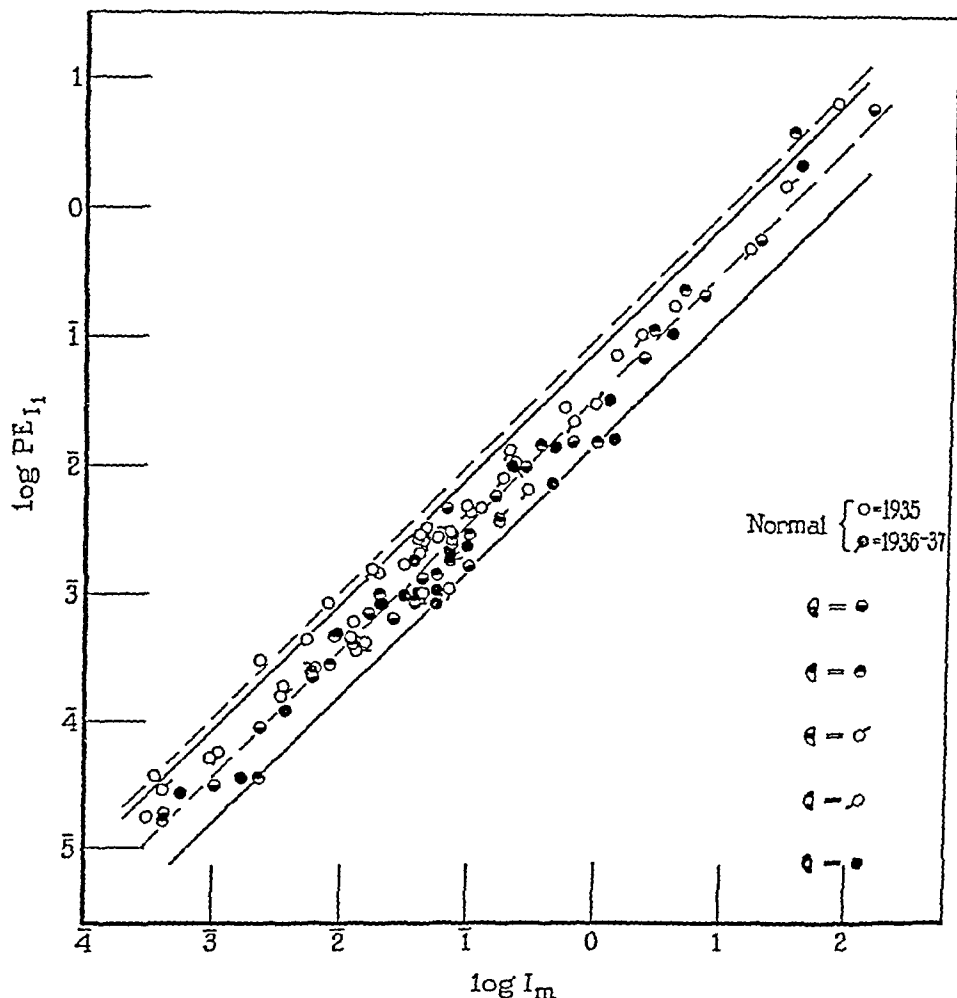
TEXT FIG. 2.  $\log F$  vs  $\log I_m$ , to show differences in the asymmetrical character of the  $F - \log I_m$  curves (Text fig. 1) produced by various partial coverings of the eyes. The least distortion is apparent in curve 5 (see text).

Sets (1) and (3) (Table I) are practically identical, as are also (2) and (4). (3) and (4) have therefore been omitted here.

no more indication of an effect upon  $\sigma_{I_1}$  than there is in the flicker response curve of normal larvae, for which the direct proportionality of  $\sigma_{I_1}$  to  $I_m$  is identical over the whole working range of  $I$  although at higher intensities the reaction is much more clean cut. The new



experiments add the important fact that with modification of the exposed area of retina the character of the response may be modified



TEXT-FIG 3  $\log P E I_1$  as a function of  $\log I_m$ . The law of direct proportionality with a slope of 1 is preserved, despite the change of the  $F - I_m$  curve produced by partial covering of the eyes. Moreover the proportionality constant is independent of the type of eye covering, so that all the data taken together (Table I) form a band of statistically constant proportionate breadth (solid lines). An older series of determinations on normal larvae of another lot (1935) gave a band of slightly higher position and of slightly less width, but the difference in breadth is not statistically significant.

both qualitatively and as to sharpness and amplitude, at the same level of  $I$ , without affecting the relation of  $I_m$  to  $P E I_1$ . This in a sense

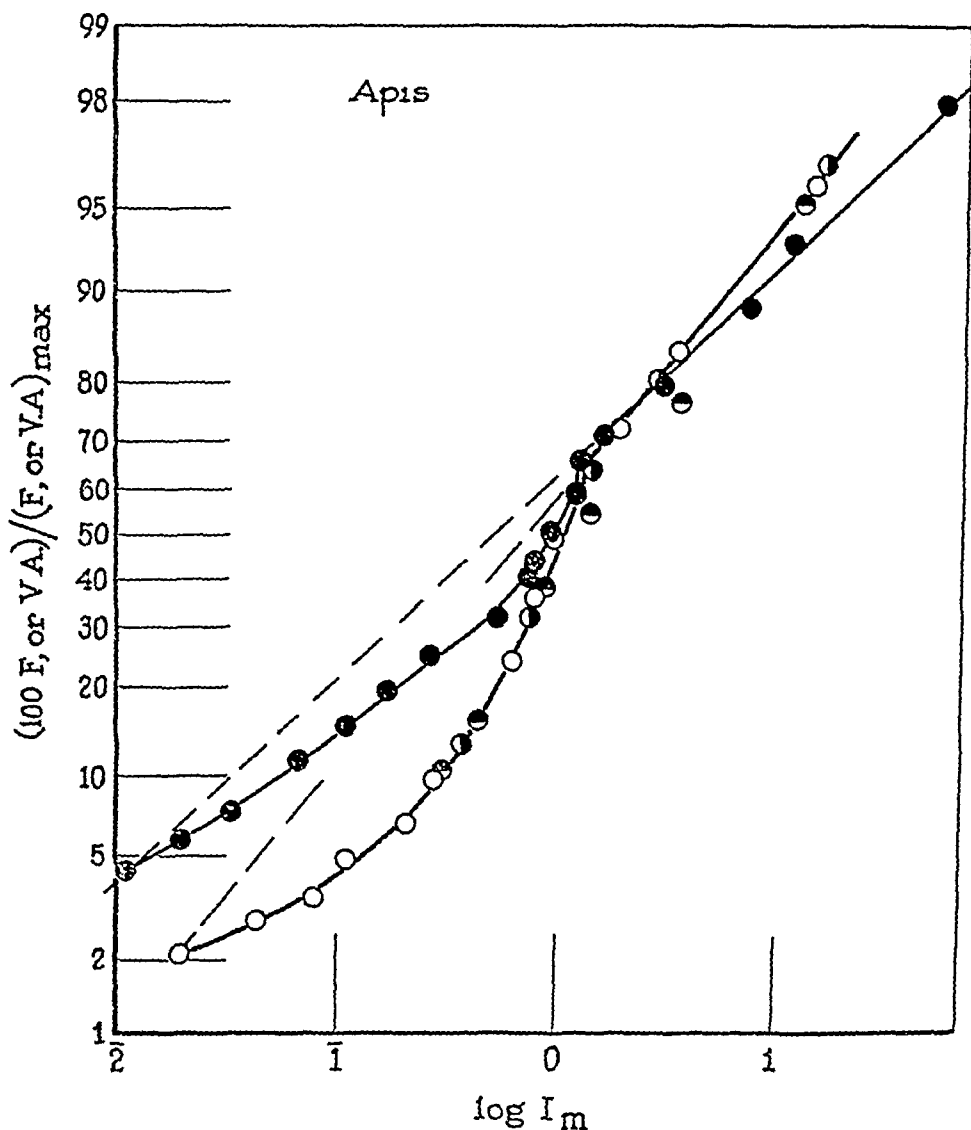
supplies the complement of experiments designed to measure intensity discrimination and visual acuity with the eyes of arthropods<sup>4</sup> (*Apis*) when the independent variable is made the width of the alternately illuminated bars of a stripe system instead of time of exposure (as in a flicker experiment), the result is essentially identical in the two cases  $k' (I_e + \text{const}) = \Delta I_m = k \sigma_{\Delta I_1}$ , and  $k$  is independent of width (area)

#### IV

The relation of  $F$  to  $\log I$  does not follow the law of a simple probability integral or population curve with the *Anax* nymph<sup>2</sup> (cf Text-fig 6) For visual responses of vertebrates tested it does<sup>11</sup> That this is not due to some peculiarity of our experimental procedure is shown by the fact that precisely similar departures from the simple rule are exhibited by data obtained with other arthropods, and by different procedures The common feature of these cases is found in the convexity and radial structure of the ommatidial surface Thus in observations upon reaction to flicker and in tests of visual acuity with the bee<sup>19</sup> a precisely similar discrepancy appears, and the departure from the simple population curve occurs at the same intensity in the two sets of measurements (Text fig 4) In acuity tests with the fiddler crab *Uca* (Clark, 1935-36) the same departure is seen (Text fig 5)

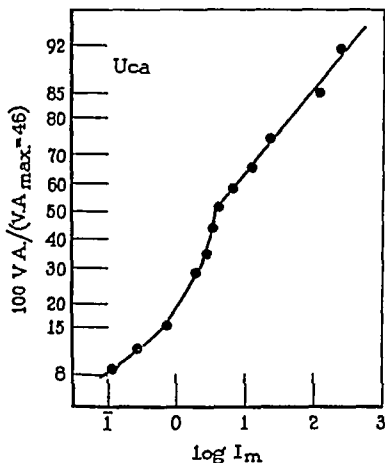
The thought arising in this connection is (Crozier, Wolf, and Zerrahn Wolf, 1936-37b) that at lower values of  $I$  certain retinal elements are unable, by virtue of their position or otherwise, to contribute to the sensory effect resulting in marginal response to flicker With higher intensities this handicap does not obtain, so that, above a certain intensity, the full complement of retinal elements is available for excitation in responses involving the discrimination of intensities Whether a given element is then actually involved at a certain moment is governed in part by the state of its own variable condition

If the disadvantage of certain elements were to be due to the existence of an asymmetrical frequency distribution of basic excitabilities, it might be expected that elevation of temperature, by bringing about an enhancement of the relative degree of dark adaptation in the photostationary state, should tend to obliterate the departure of the



TEXT-FIG 4 A population integral plot of data on  $F - \log I_m$  for the bee (solid circlets), and for visual acuity ( $V A$ ) data for the bee (open and partially shaded circlets) obtained by quite different procedures<sup>19</sup>. As with flicker measurements on *Anax*,<sup>3</sup> there is a sharp discontinuity at about  $F$  (or  $V A$ )/ $F_{max}$  (or  $V A_{max}$ ) = 67–68 per cent (with *Anax*, 74 per cent). The projection of the line fairly describing the upper portion of the data passes through the lowest point, as also with *Anax*.<sup>3</sup> The half-shaded circles refer to  $V A$  measurements<sup>19</sup> with bee's eyes vertically divided by an opaque covering, the form of the function is not especially affected.

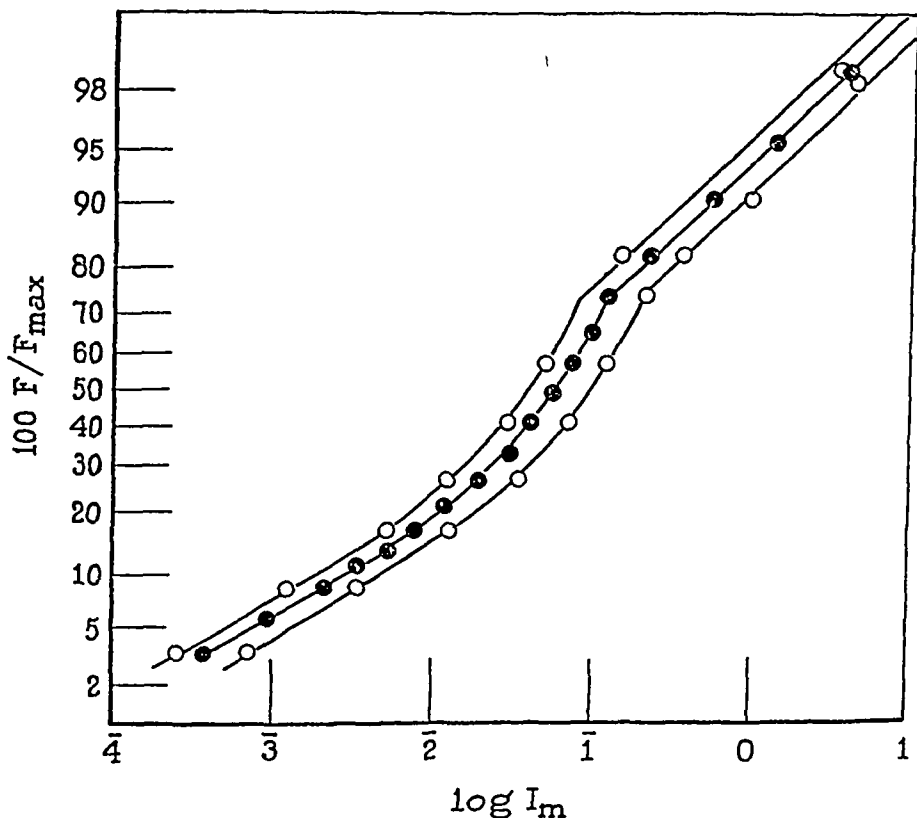
flicker data from a population curve. A decrease in the proportion of flicker cycle time occupied by light, however, should accentuate the departure of  $F$  vs  $\log I_m$  from a population integral if not enough light is being admitted during a flash to ommatidia in a disadvantageous position. The latter effect is indeed obtained.<sup>7</sup> The relation to temperature is such that (Text fig. 6) there is no real indication what



TEXT FIG. 5 Data from measurements of visual acuity by Clark (1935-36) with the fiddler crab *Uca* show that for the convex eyes of this arthropod also the same type of population curve is obtained as in Text figs 4 and 7 ( $V A_{max} = 46$ )

ever that the presumed disadvantaged receptors have been put in a relatively more favorable state by raising the temperature, changing the temperature from 12.4° to 27.3°C has merely decreased  $\log I_m$  by about 0.4 units at all values of  $F$ . This is exactly the nature of the result when the temperature is altered with the sunfish.<sup>2</sup> The two results, taken together, signify that some largely mechanical or structural condition limits the activity of certain ommatidia, rather than

that an intrinsically distorted frequency distribution of effective thresholds or excitabilities is responsible for the effect. In the normal *Anax* larvae (*i.e.*, with unpainted eyes), certain ommatidia do not contribute at all to the determination of response until fairly high intensities are used. The first derivative of the  $F - \log I_m$  curve is



TEXT-FIG 6  $100 F/F_{max}$  vs  $\log I_m$  for normal *Anax* larvae at three temperatures<sup>3</sup>—27.3°, 21.5°, 12.4°—on a probability grid ( $F_{max}$  for 12.4° = 61.1, for the others, 60.9). The form of the curve is not affected by temperature, for a given change of temperature  $\log I_m$  is decreased by a constant amount at each value of  $F$ .

taken to give the frequency distribution of  $\log I$  thresholds for effective contributions. When reduced to the same area (*i.e.*, to  $F_{max} \equiv 100$  per cent) the curve for larvae with eyes having only the central area exposed is the most nearly symmetrical. Consequently the disadvantaged units must be regarded as those situated near the periphery

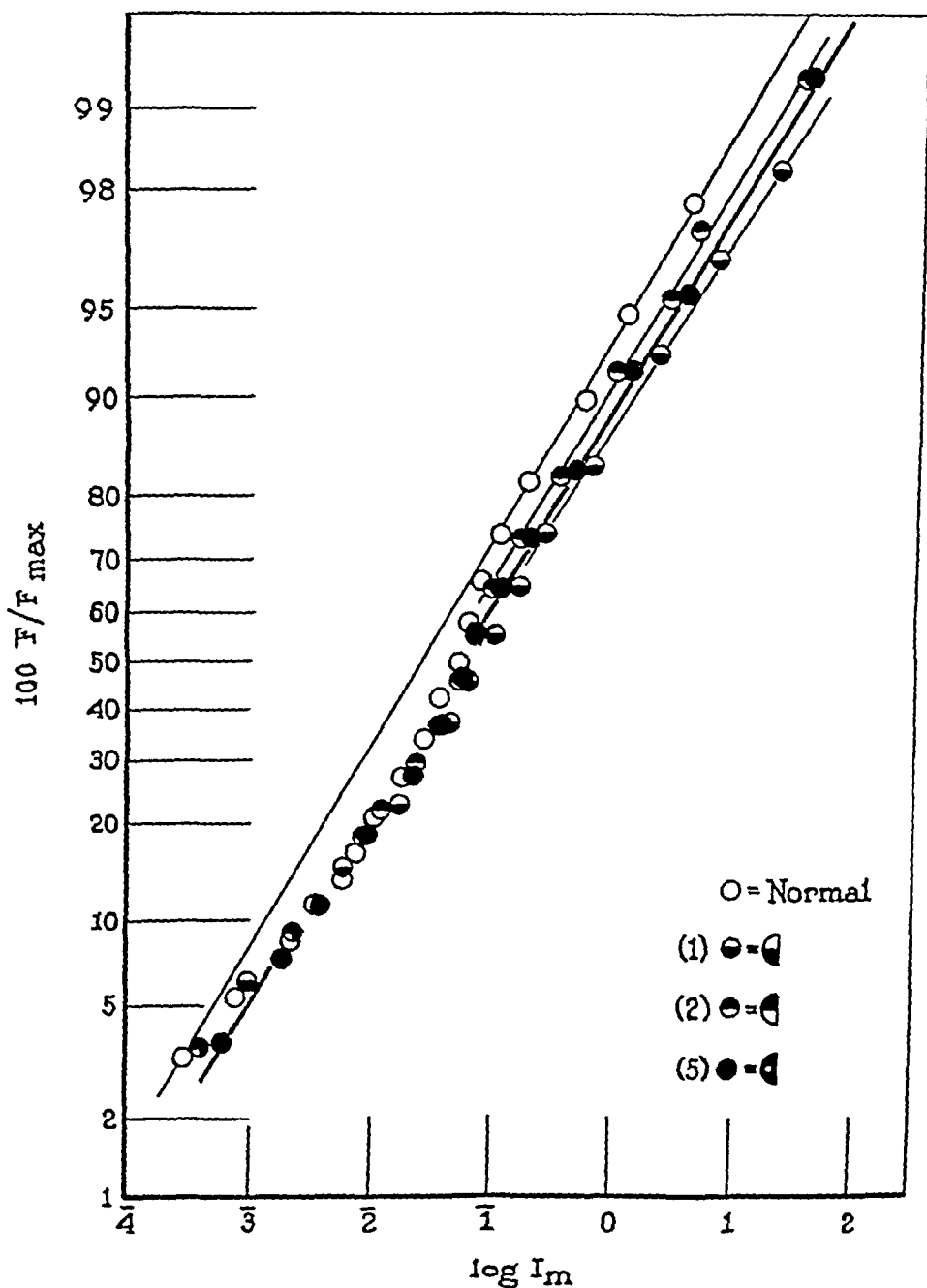
of the optic surface. The argument for regarding  $dF/d \log I$  as a frequency distribution in terms of  $\log I$  is derived (1) from the probability integral character of the  $F - \log I$  curve and (2) from the properties of  $\sigma_{1r}$  and of  $\sigma_{1r}$  as a function of  $\log I_m$  (cf Crozier, Wolf, and Zerrahn Wolf, 1937-38a).

Even with only the central area of the eye exposed the graph (Text figs 1 and 7) is not perfectly symmetrical. This might be due in part to real and persisting regional differences in sensitivity within the small area concerned, although this is less than one fifth of the total ommatidial surface. We do not consider this very probable, however. It could also be suggested that the apparent residual asymmetry might be due to the fact that the lower asymptote of the curve should not be 0, but a slightly larger number. Although on this basis, assuming for example that  $F_0 = 1.5$ , it is possible to secure a statistically satisfactory fit<sup>20</sup> with a probability integral, we consider this explanation unlikely. A more reasonable interpretation is to be found in the fact that the margins of the lacquer do not provide a perfect cut-off for light, with higher intensities, effective light leakage, involving a slightly larger number of ommatidia, becomes increasingly potent. This does not contradict the probability that the central area of the eye is sensorially more effective than the peripheral edge, but it does not imply that the individual ommatidium differs markedly from one region of the eye surface to another, since in the central area they are smaller and more densely grouped.<sup>21</sup> This condition is consistent with the fact that the maximum  $F$  attained is only slightly less with the central area alone than with the normal eye. If it be assumed then, that light leakage under and through the edge of the opaque covering modifies the extent of the excitable area, it might be possible to correct for this (in the case of the centrally exposed eye) by suitable assumption of an arbitrary ideal value of  $F'_{max}$  for this area alone, the curve should then fit the lower portion of the  $F - \log I_m$  data, but there is no adequate test for this procedure.

It is reasonable to suppose that at low values of  $F$  (and thus of  $I_e$ )

<sup>20</sup> Satisfactory in the sense that the departures ( $v$ ) at any point are not greater than  $PE_{1r}$  or greater than  $3 \times PE_{1r}$ , the departures are systematic, nonetheless since the mean value of  $v/\sigma_{1r}$  is greater than 0 and its  $SD > 1$ .

<sup>21</sup> Cf Baldus, 1926; del Portillo, 1936.



TEXT-FIG 7  $F/F_{max}$ , on a probability grid, for *Anax* larvae, normal and with parts of the eyes covered. For the normals,  $F_{max} = 60.9$ , for (1), 53.9, (2), 54.5, (5), 54.4. (As in Text-fig 2, (3) and (4) have been omitted, since they are practically identical with (2) and (1) respectively.) The departure from rectilinearity is least with (5).

only a small number of retinal elements require to be excited for the recognition of flicker. The area from which they are to be obtained will be determined by (1) the amount and the location of the uncovered surface of the eye, and (2) by the posture of the animal in relation to the striped cylinder. Hence the curves for various types of partial covering of the eyes all tend to run together at the lower end. Larvae with the anterior half of the eye covered show an accentuation of the features of the normal curve in that the departure of the lower part from the ideal population curve is slightly greater and the upper part is moved to higher intensities. The presence of a vertical bar of lacquer has about the same effect, absolutely and relatively, as does the covering of the anterior half (Text figs 1, 2, and 7). The asymmetry is decreased by painting out the posterior half of the eye, or to the same extent by allowing only a vertical band in the center to be uncovered. The asymmetry is least when only the central area is exposed (Text figs 1, 2, and 7). Thus the chief reason for the departure from a simple population curve for  $F - \log I_m$  is found in the activity of ommatidia around the edge of the eye. The behavior of the  $F - \log I_m$  curve clearly implies that the most deficient region of the normal eye, in flicker response at low intensities, is on the posterior part of the ommatidial surface.

Obviously, it is not completely demonstrated by these findings that the posterior retinal elements are intrinsically less irritable. Part of the complication is due to the position assumed by a larva with reference to the striped cylinder in the situation provided by the procedure we employ. If a technique could be applied adequate to disclose the individual thresholds for flash excitation, a quite different picture of the spatial distribution of ommatidial excitabilities might very well be obtained.

We have been concerned specifically with the explanation of the special form of the  $F - \log I_m$  curve for *Anax* nymphs. Thus we are not required at the moment to examine the question as to whether the "most sensitive" part of the eye as it might be revealed by orientation tests, or by electrical phenomena, or by determinations of photic thresholds, is necessarily the region predominantly contributing to the flicker recognition process. The qualitative indications, however, are that the central zone is the most effective. The occurrence of similar properties in the curves of visual response obtained with other



arthropods supports the conception of a purely mechanical origin of the "distortion" of the *Anax* flicker curves for which the results of partial covering of the eyes provide a consistent explanation. In the normal eye, under our conditions, as  $F$  is made greater, a certain number of elements hitherto unconcerned in the determination of the response begin to be involved, especially just beyond the inflection point of the  $F - \log I_m$  curve. In larvae with anterior or posterior halves of the eyes covered the *proportion* of the elements stimulated with comparative difficulty is greater, hence the asymmetry of the curve is increased. In those with a vertical opaque stripe over the center of the eye there is only a slight reduction of the asymmetry, easily explained by the posture assumed by these animals. With the central stripe of the eye uncovered and the front and back regions opaqued, there is a considerable reduction of asymmetry. Hence the periphery of the eye must be the region responsible for it, and especially the posterior half. Consequently it is not surprising to find that with the margin of the eye completely covered the asymmetry is least. Were it possible to provide a rigid isolation of parts of the eye surface by applied coatings, the curve could presumably be made quite symmetrical.

The properties of  $\sigma_r$  as a function of  $I_m$  show, since the relationship is quite independent of the area or the region of the eye excited, that response to flicker is independent of the peripheral point of excitation and is governed by a uniform general mechanism. This mechanism is of course dependent upon the primary peripheral excitation. In terms of the probability integral formulation we may deduce from Text-figs. 1 and 6 that  $F$ , as a measure of sensory effect, is in this case in part determined by the total receptor area exposed, in part by the native excitability of this area or part under the conditions of the test. Accepting the probability curve as fundamentally correct despite mechanically produced distortion at lower intensities of illumination, we may compare the regional excitabilities in terms of two parameters (*cf* Crozier, 1937) (1) the standard deviations ( $\sigma_{\log r}$ ) of the presumptive underlying (percentage) frequency distributions of excitabilities in the elements connected with the several areas and (2) the values of  $\log I_m = \tau'$  for which  $F/F_{max} = 50$  per cent. The second parameter should eliminate the mere influence of total number

of elements as a factor in each area. The values of  $\sigma_{\log I}$  are practically the same for all the areas tested. This agrees with the indications of the visual acuity data on *Aphis*, already cited. The values of  $\tau'$  increase in the order (i) *normals*, (ii) *posterior half open*, and *central bar covered*, (iii) *central spot open*, (iv) *anterior half open*, and *central stripe open*.

There is no indication here that individual excitation elements in different parts of the eye, as concerned in excitation by flicker, exhibit marked differences in threshold or in capacity to contribute to the critical degree of excitation. If anything, the anterior half of the eye, in view of the posture involved (section II), shows a slightly wider range of relative excitabilities, but its higher  $\tau'$  is only a little greater than that for the central circlet on the eye. Using  $\tau'$  as criterion, the regional order of decreasing excitability for areas giving  $F_{max} = 100$  per cent is *posterior half, central region, anterior half*.

The quantity  $\tau'$  is the homologue of the chronaxie  $\tau$  in an electrical excitation experiment (Crozier, 1937). In a voltage duration curve the reciprocal of the voltage  $V$  (or current) corresponds to  $F$ , and  $I$  to  $t$ . We have seen reason to regard  $F$  as a proportionate measure of intensity of sensory effect  $E$ , if time is not involved in the measurements,  $E$  is also measured by  $1/V$ . The parallelism between the properties of the flicker curves and the phenomena of the strength duration curves is striking. In tests of electrical excitability involving experimental treatment of the tissue it happens that an increase of rheobase ( $V_0$ ) is frequently accompanied by a decrease of chronaxie, a result which has been regarded as perplexing. In the present tests with flicker a reduction of  $F_{max}$  is obtained by reducing the area of retina, accompanied by an increase of  $\tau'$ . Reduction of  $F_{max}$  means an increase in the lower asymptotic level of  $1/F_m$ , equivalent to an increase of  $V_0$ . This is seen to be a consequence of the fact that the number of sensory elements open to stimulation has been reduced by elimination of some of the more easily excited. This clearly corresponds to the case when, for example, with a nerve the inter electrode distance is shortened, or when certain drugs are applied, or when the temperature is lowered, in the latter instance  $\sigma_{\log I}$  (like  $\sigma_{\log I}$  in the flicker experiments<sup>2</sup>) is not affected, but in the first two the frequency distribution of excitabilities of elements is usually changed by

the procedure, so that  $\sigma_{\log I}$  is increased. The fact that in the flicker experiment  $\sigma_{\log I}$  is not much changed by reducing area shows that the factor governing accessibility to excitation is not a matter of intrinsic differences of irritability in different parts of the optic surface, the ommatidia of the normal *Anax* eye do not compose a frequency distribution of irritabilities with a spatially fixed pattern.

#### SUMMARY

Arthropods with large convex eyes provide curves of critical illumination for response as a function of flicker frequency (or of visual acuity) which depart from the probability integral type characteristically found for  $F - \log I$  with vertebrates. By means of experiments with *Anax* nymphs in which various parts of the eye have been opaqued it is shown that the special shape of the flicker curve is due to the mechanical disadvantage of the periphery of the eye in the reception of light, which is overcome by higher intensities. It is not due to a fixed spatial pattern of intrinsic individual excitabilities of the ommatidia.

Reduction of retinal area decreases  $F_{max}$ , and increases  $\log I$  for  $F/F_{max} = 50$  per cent. The direct proportionality of  $I_m$  to  $PE_{1f}$  is independent of area. Certain relations of these facts to the theory of response to flicker have been discussed.

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## EXPLANATION OF PLATE 1

FIGS 1-5 Photographs of anterior end of *Anax* nymphs showing eyes prepared by covering a part with opaque enamel





3

4



5

(Crozier et al. Cr. t. callum n. t. with f. l. ae) <



# A PHASE RULE STUDY OF THE PROTEINS OF BLOOD SERUM

## III GLOBULIN\*

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(Accepted for publication July 15, 1937)

### INTRODUCTION

For many years the authors have been engaged in determining the composition of phases which occur in the system, globulin, salt, and water. In this communication a new method is described for applying Gibbs' phase rule to the characterization of the serum globulins as single proteins. The method may be applied to a study of a system of any number of protein components. It takes a small fraction of the time usually needed to carry out the experiments. Experience of our own and others has shown that the methods previously used fail to prove these globulins to be homogeneous substances. The principal difficulties found by other investigators have been overcome by keeping the protein to be studied in its original form in the medium in which it occurs.

It had been observed by several early workers that the amount of globulin remaining in solution after a definite amount of salt had been added to its solution is dependent upon the concentration of the protein in the original mixture (1). There was, as they said, a fractionation taking place. If one considered the protein as a single substance the phase rule did not apply, or considering Gibbs' phase rule to hold with proteins, globulin was not a single substance (2).

The application of this procedure has not only shown that the phase rule may be used to show that certain globulins are homogeneous substances but may also be used for a like study of each protein component.

\* This work was aided by The Rockefeller Foundation Fluid Research Fund



## EXPERIMENTAL

*Early Work*

In 1930 an attempt was made to indicate the general nature of the phase rule equilibria obtainable and reference may be made to the diagram then published (3)

More detailed studies of portions of the phase rule diagrams for horse serum and rat serum are given in Figs 1 and 2 (4)

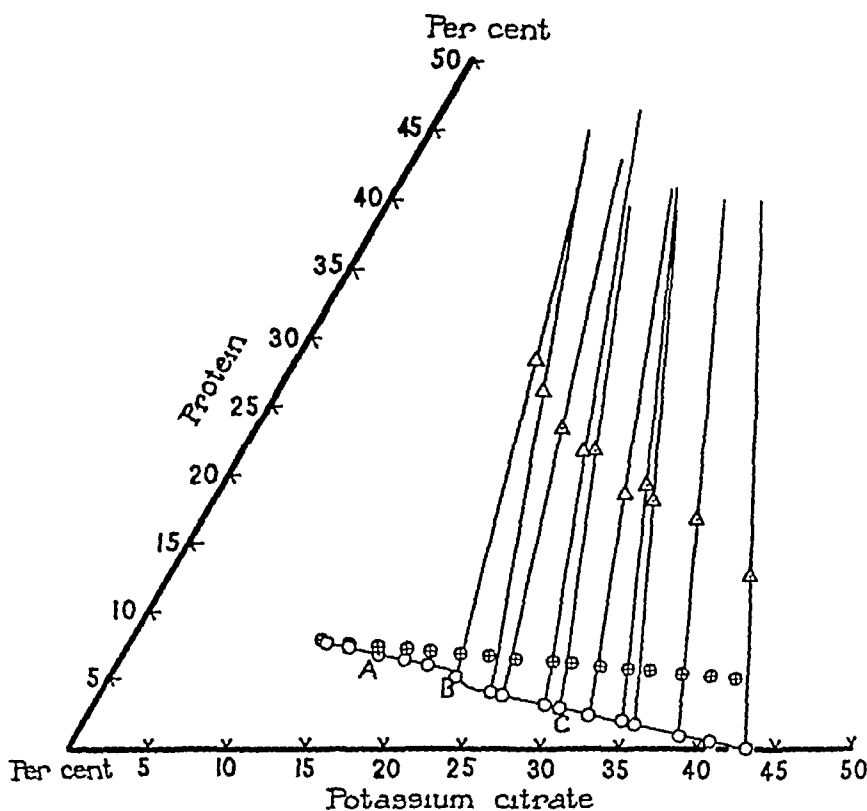


FIG 1 Horse serum

⊕ = total composition

○ = liquid phase

△ = solid phase

The results are plotted as per cent by weight on Gibbs' triangular phase rule diagrams. Pure protein, pure salt, and water are to be found at the apices of the triangles. Only a portion of the diagram is given as may be seen from the percentages along its sides.

A is considered the first fraction to appear as a solid phase on adding

potassium citrate, B is then precipitated on further addition, coming down in a very narrow range of salt concentration, and C is precipi

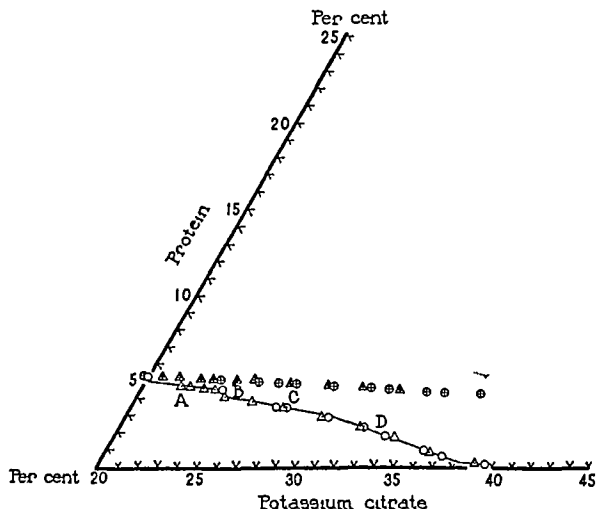


FIG 2 Rat serum Composite curve from two pools of rat serum from 30 rats each

- ⊕ = total composition, pool 1
- = liquid phase, ' 1
- △ = total composition, ' 2
- △ = liquid phase, ' 2

tated gradually as the salt concentration becomes higher. Finally D, an albumin fraction, appears in some serums<sup>1</sup> (Fig 2)

<sup>1</sup> The experimental procedure used in these experiments was described in a previous communication. To weighed portions of dialyzed serum or protein solutions at pH 6.8, potassium citrate was added to make the desired salt concentration. The pH was maintained by added citric acid. The precipitated protein was separated on filters and the liquid phase analyzed for protein and potassium. All analyses were made on weighed samples—so the results are in per cent by weight. All processes were carried out as rapidly as possible at 0°C.

A clear illustration of the application of the phase rule may be seen in Figs 3 and 4 (4) One fraction after another has been salted out from the rat serum by increasing the potassium citrate concentration. The curves of undiluted and diluted serum converge showing that the protein in solution is independent of its concentration in the

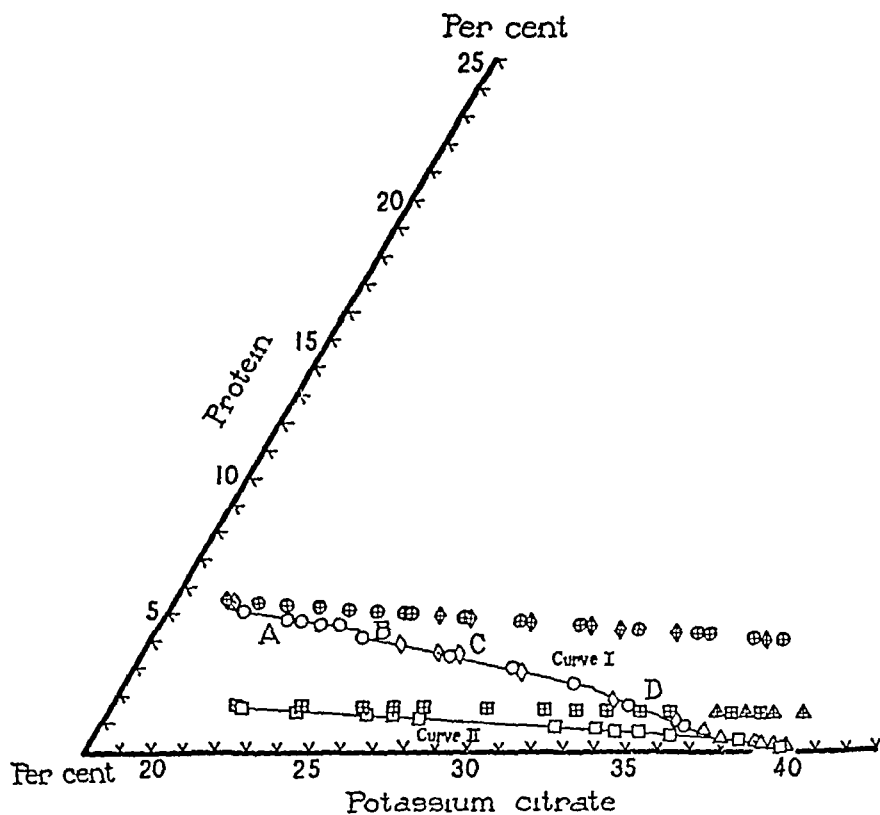


FIG 3 Comparison of diluted and undiluted rat serum

- ⊕ and ⊞ = total compositions, undiluted
- and ◇ = liquid phases, undiluted
- ⊞ and △ = total compositions, diluted
- and △ = liquid phases, diluted

mixture at high salt concentrations. Enough solid phases are present to fix the concentration of the protein in solution.

Preliminary experiments were made on globulin prepared from horse serum by the method described by Svedberg. A phase rule study was carried out with this substance,  $(\text{NH}_4)_2\text{SO}_4$ , and water.

An early experiment showed a definite solubility of this globulin fraction in the same concentration of  $(\text{NH}_4)_2\text{SO}_4$  regardless of the amount of protein in the mixture. In this case the protein precipitated from a solution of globulin was added to another portion of the original solution to make the concentrated solution (3).

New experiments were tried under a variety of conditions in an

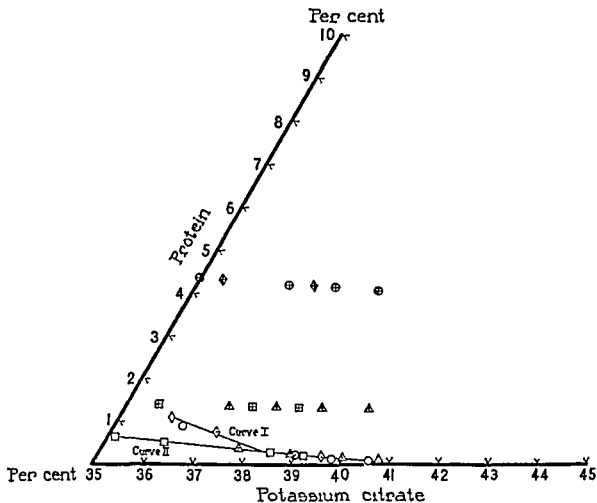


FIG 4 Enlargement of a section of Fig 3

effort to preserve globulin in its original form. They differed from previous experiments since in all cases comparisons were made between concentrated solutions and others diluted from them. Horse serum globulin which had been precipitated by half saturation with  $(\text{NH}_4)_2\text{SO}_4$ , washed repeatedly, reprecipitated, and rewashed twice, was used for the concentrated solutions. Figs 5 and 6 give illustrations of the data. As may be observed in Fig 6 when the total

composition represents 10.5 per cent of protein and 14.8 per cent salt, the liquid phase separating contains 4.6 per cent protein. When the total compositions on the same tie line show 8.4, 6.25, and 2.0 per cents protein respectively, the liquid phases contain 3.7, 2.9, and 1.05

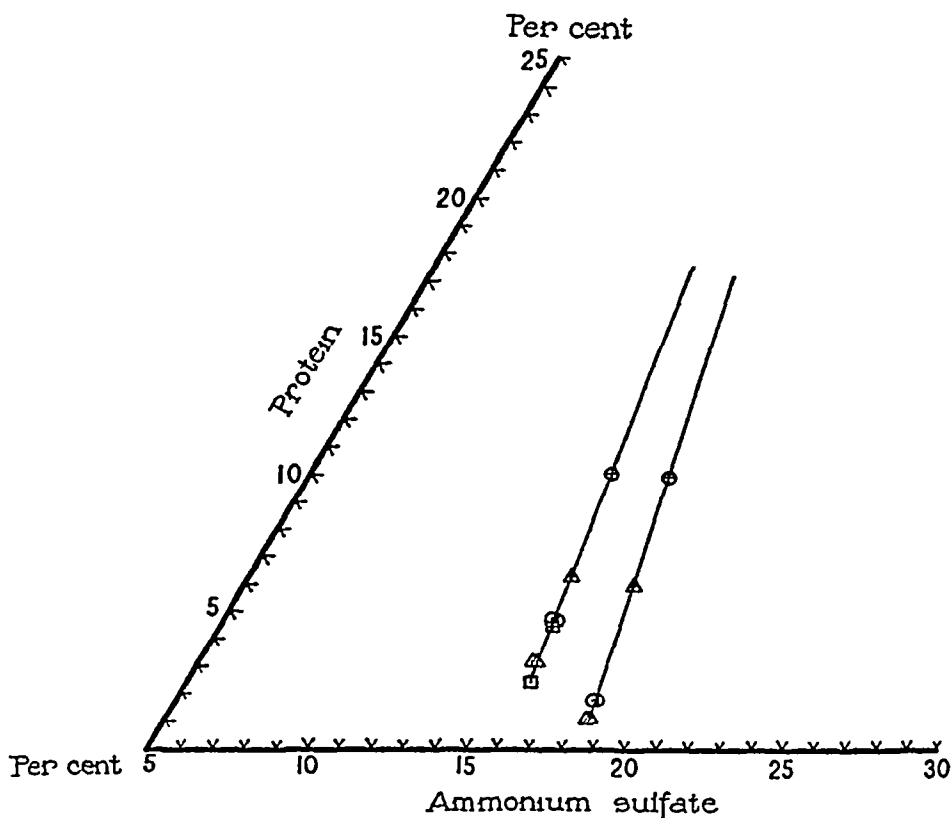


FIG 5 Globulin separated by ammonium sulfate

- ⊕ = total composition, concentrate
- = liquid phase, concentrate
- △ = total composition, diluted sample 1
- △ = liquid phase, " " "
- ⊞ = total composition, " " 2
- = liquid phase, " " "

per cents protein. No matter what the conditions of the experiment (changes in temperature, etc.) the results were the same. The observations of earlier workers on the dependence of the solubility of globulin on the amount in the mixture under these conditions were corroborated. An explanation of these apparently conflicting results

will appear in the discussion (The failure to understand these observations at the time led to a study of the whole salting out curve of a very fresh undiluted serum at  $0^{\circ}\text{C}$ , to make certain how many globulin fractions were present in the native state)

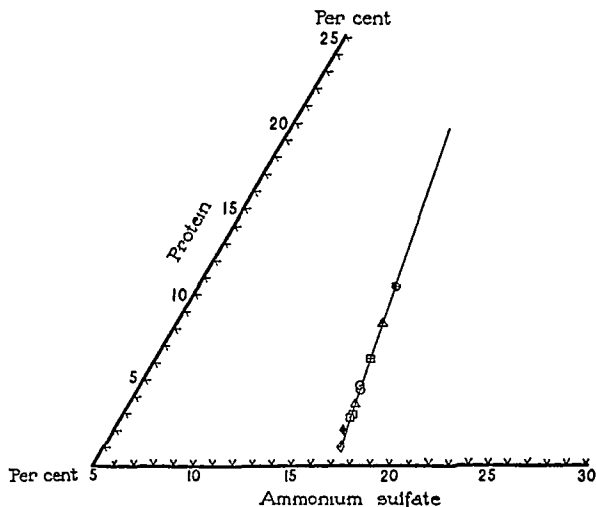


FIG 6 Globulin separated by ammonium sulfate

- ⊕ = total composition, concentrate
- = liquid phase, concentrate
- △ = total composition diluted sample 1
- △ = liquid phase " "
- ⊞ = total composition, " 2
- = liquid phase " "
- ⊕ = total composition, diluted sample 3
- ◇ = liquid phase " " "

Early experiments confirmed observations already made by Hardy (5) The fraction precipitated by saturation with  $\text{NaCl}$  proved to be extremely unstable, separating readily into a soluble fraction and a

highly pigmented insoluble one. Ammonium sulfate seemed to have a deleterious effect on one of the globulin fractions. In consequence, the method of precipitation with potassium citrate was developed.

The proportion of the total serum protein precipitated by 31 per cent citrate is similar to that precipitated by half saturated  $(\text{NH}_4)_2\text{SO}_4$  solution (Fig 1). It is obvious that there are at least two changes in direction with increasing salt concentration up to 31 per cent citrate, indicating three different fractions separating. From a statement of the phase rule we could not expect to have a constant per cent of protein in solution when keeping the salt concentration and temperature constant and varying the protein until there were three precipitated phases, or until the salt concentration was nearly 31 per cent of potassium citrate or half-saturated with  $(\text{NH}_4)_2\text{SO}_4$ . It was in such a part of the curve with  $(\text{NH}_4)_2\text{SO}_4$  after nearly all globulin was removed from salt solution, that the point of constant solubility was found in our first experiments.

In the results with salting out of fraction "A" with potassium citrate, it was noted that the more of this fraction present, the lower the salt concentration at which it began to separate. This fact suggested a definite solubility. A comparison may be made with the albumin fraction "D" in Figs 3 and 4. It is evident that it does not begin to separate on addition of salt until its point of saturation is reached. Of course, when the concentration of D is less a higher salt concentration is required for its first appearance on the salting-out curve.

During some experiments carried out by Dr T. Addis on the effect of diet on body proteins of rats, depletion of the proteins was produced by fasting and then restored by refeeding proteins. The blood serum from these rats was studied. On regeneration certain protein fractions were replaced before the others, one of these being a globulin fraction C'. In newborn calves Howe (6) had reported that the globulins were formed very quickly upon feeding colostrum. We decided to study these fractions, particularly as it was observed that the globulin of calf serum so rapidly formed was anisotropic and had a definite crystalline form. Experience had proved to us that certain fractions of globulin are extremely unstable. Therefore, it seemed essential, in so far as it was possible, to study them without removing

them from the original serum. This idea was carried out by dissolving protein which had been precipitated from serum by a certain per cent of potassium citrate in another portion of the same serum and then comparing the solubility curves of the serum and the concentrate so made. If the phase rule applies in its usual form and if only the first fraction, A, has been added, precipitation should begin at a lower

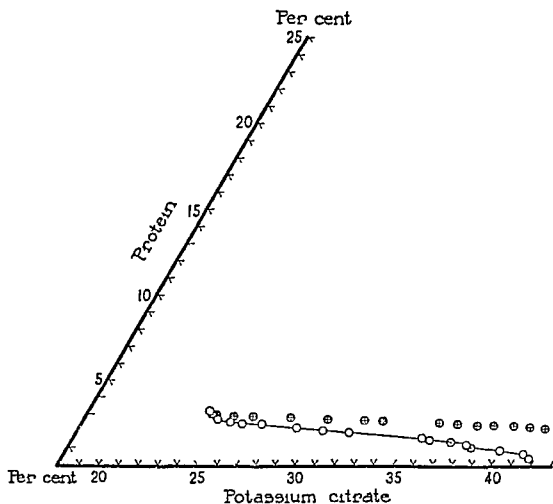


FIG 7 Newborn calf serum In Figs 7 and 8

⊕ = total composition

O = liquid phase

salt concentration in the concentrate than in the serum and the two curves should coincide beginning with the point of first precipitation of A in the serum. The thermodynamical potential of the phases would not have been changed. If A and B have been added the solubility curve of the latter part of B and subsequent fractions should coincide, but not that of A.



A solubility curve with potassium citrate was made from the blood serum of a newborn calf Fig 7 shows that precipitation does not begin until 24 per cent citrate has been added The first two fractions to be precipitated from other serums, namely A and B, are not visible in this calf serum (See Howe )

Fig 8 illustrates the effect of feeding colostrum for 2 days to a newborn calf Since precipitation begins now at 19 per cent citrate it is

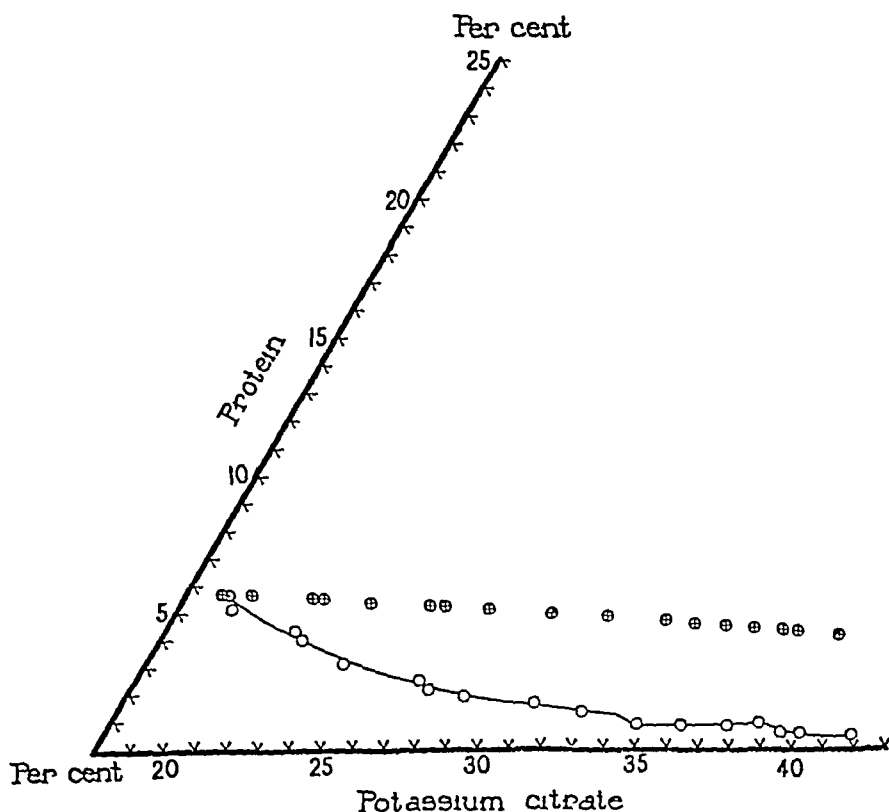


FIG 8 Colostrum fed calf serum

evident that fraction B has been formed rapidly From the appearance of this curve the fraction up to 26 per cent citrate was provisionally assumed to be a single protein

#### *Application of the Phase Rule*

Another calf was fed colostrum for 2 days and its serum globulin studied In order to maintain the concentration of the other proteins

constant, unwashed globulin precipitated from 240 cc of serum at 26 per cent citrate was added to the 100 cc of the original serum. In this calf serum protein production had proceeded further and fraction A had appeared as seen in Fig 9. The curve is drawn to follow the direction of that in Fig 7 at 23-24 per cent citrate although the points

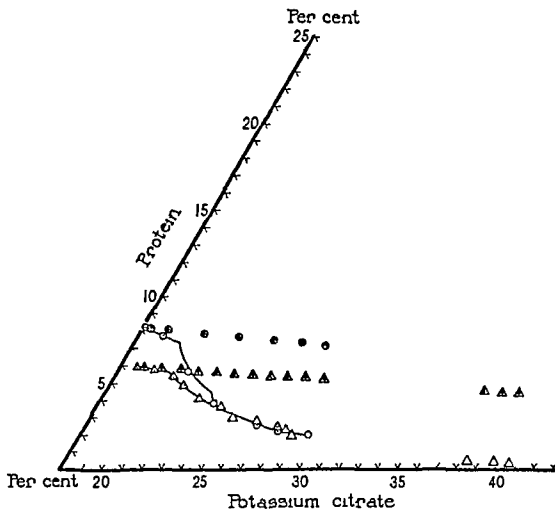


FIG 9 Colostrum fed calf serum

- $\oplus$  = total composition, serum with added globulin
- $\circ$  = liquid phase, " "
- $\blacktriangle$  = total composition, serum unchanged
- $\triangle$  = liquid phase, " "

are not close enough to determine the line. It is now apparent that the break in the continuity of the line is between 23 and 24 per cent instead of at 26 per cent. When the precipitate made at the latter salt concentration was added three fractions A, B, and C were added. Theoretically the curves should then coincide at about 24 per cent if

the proteins are homogeneous. This actually took place. If C had been fractionated by precipitation from the original serum, the more insoluble precipitated part should have displayed a different solubility from the original C and there would be a divergence up to 26 per cent

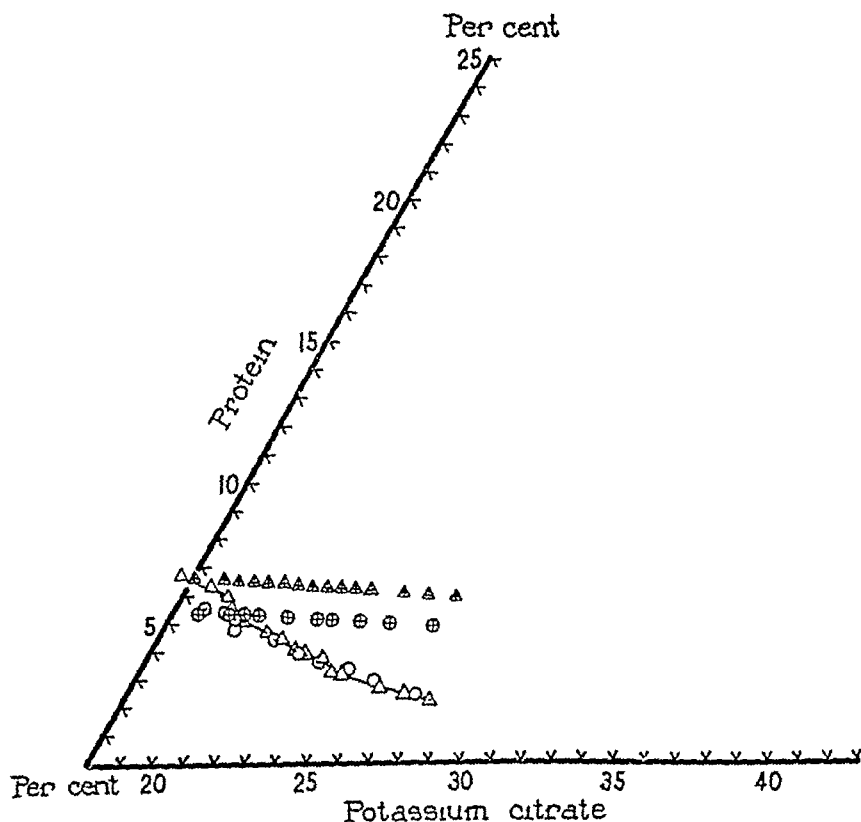


FIG 10 Colostrum fed calf serum

- △ = total composition, concentrate I
- △ = liquid phase, " "
- ⊕ = total composition, unchanged serum
- = liquid phase, " "

or a different curve entirely along the line of C, unless after fractionation the same equilibrium had been established

Serum from another colostrum fed,  $2\frac{1}{2}$  day old calf was utilized in the next experiment. The precipitate from 90.4 gm of serum at 24.15 per cent potassium citrate dissolved in 100 cc of serum formed con-

concentrate No 1 During dialysis of this solution and the original serum some dilution took place, unfortunately slightly more in the concentrate than in the serum The coincidence of the two salting out curves, compared in Fig 10 is obvious although not perfect There is an appreciable divergence only in the part of the curves representing the precipitation of A and the first part of B Both A and B begin to precipitate at lower salt concentrations in the concentrate than in the serum There is no evidence whatsoever of anything like a proportionality between the protein in the original mixture and that remaining in solution as may be seen from comparing the points of total composition of the two series Protein is precipitated from the concentrated solution until the concentration of the protein in the liquid separated from the precipitate is equal to that of the serum Here the two curves join Comparing the curve made from concentrate I with that made from concentrate II (Fig 11) formed by dissolving protein precipitated from 224.5 gm of serum at 21.4 per cent potassium citrate in 60 cc of the same serum, the salting out curves are found to be in the main part parallel although the protein content of the two concentrates is very different Concentrate I would contain unfractionated A and B if the assumption is correct that practically all of A and B have been precipitated at 24.15 per cent of citrate The precipitation of A undoubtedly overlaps that of B Since a greater volume of the serum was precipitated for concentrate II than for concentrate I, there is a greater concentration of A in the former Also at 21.4 per cent citrate only part of the fraction B has been precipitated and added to make concentrate II If a fractionation took place during the precipitation of B only the most insoluble fraction could have been added There is no proportionality between the per cents of protein in the mixtures and those in the solutions The mixtures with higher per cents of total protein (concentrate II) give solutions with less per cents of protein at the same potassium citrate content This is because concentrate II is more dilute with respect to C and the fractions of protein precipitating at greater concentrations of citrate than is concentrate I The convergence of the curves in Figs 9 and 10 at greater concentrations of potassium citrate than 24 per cent is evidence of this fact When the

concentrations of C were the same, the solubility curves of B coincided (Fig 10) The solubility curve of A in the second concentrate is not well defined but probably falls sharply (Fig 11)

Horse serum globulin was studied in the same manner as the calf serum globulin Potassium citrate was added to the serum to make

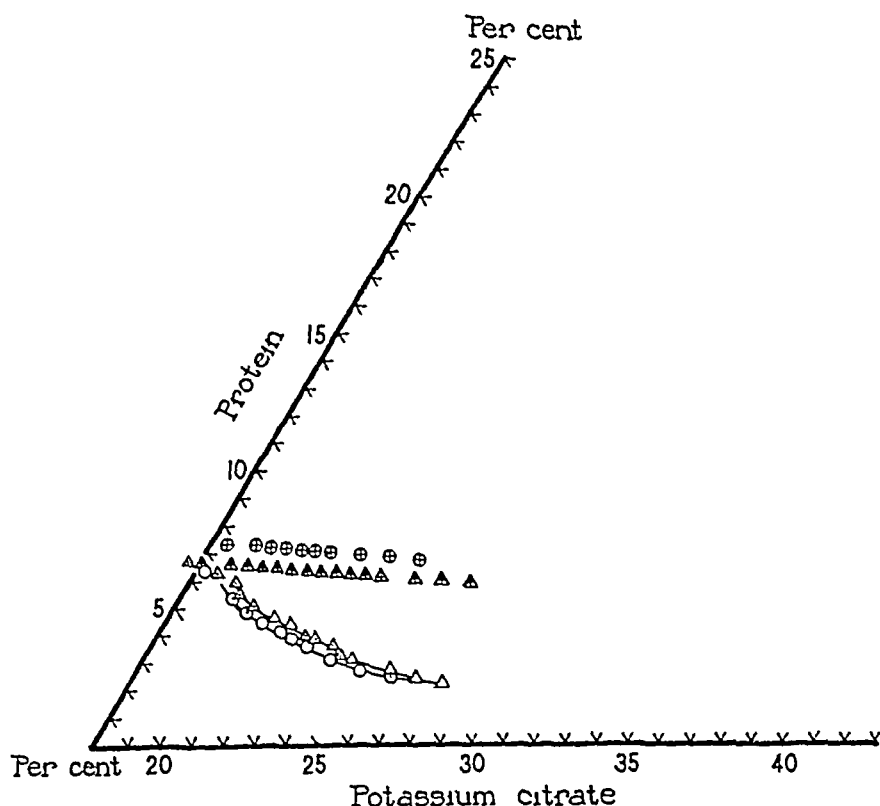


FIG 11 Colostrum fed calf serum

- △ = total composition, concentrate I
- △ = liquid phase, " "
- ⊕ = total composition, " II
- = liquid phase, " "

at 20.8 per cent citrate. The precipitated fraction, separating, was freed from the greater part of its adhering liquid by being pressed between filter paper. It was then dissolved in serum to make two concentrates of different strengths (I and II). Citrate was added to the above filtrate to bring about a second precipitation at 23.5 per

cent citrate This second precipitated fraction was now partially dried with filter paper and added to small portions of the first filtrate to make concentrates III and IV

TABLE I

	Protein	Potassium citrate
	<i>per cent</i>	<i>per cent</i>
Concentrate I	12.8	7.33
Concentrate II	14.3	8.50
Concentrate III	9.95	15.04
Concentrate IV	12.0	16.6

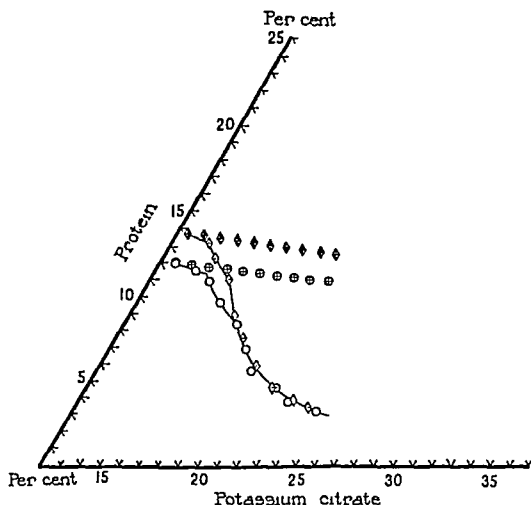


FIG 12 Horse serum

- ⊕ = total composition, concentrate I  
 ○ = liquid phase, " "  
 ⊕ = total composition, II  
 ◇ = liquid phase, "

To several weighed portions of each of these four solutions potassium citrate and citric acid were added as usual at  $0^{\circ}$  and pH 6.8 and the filtrates from the precipitates analyzed. From these analyses the curves in Figs 12 and 13 were constructed. The curves have the same general nature as those from calf serum. Those from concentrates

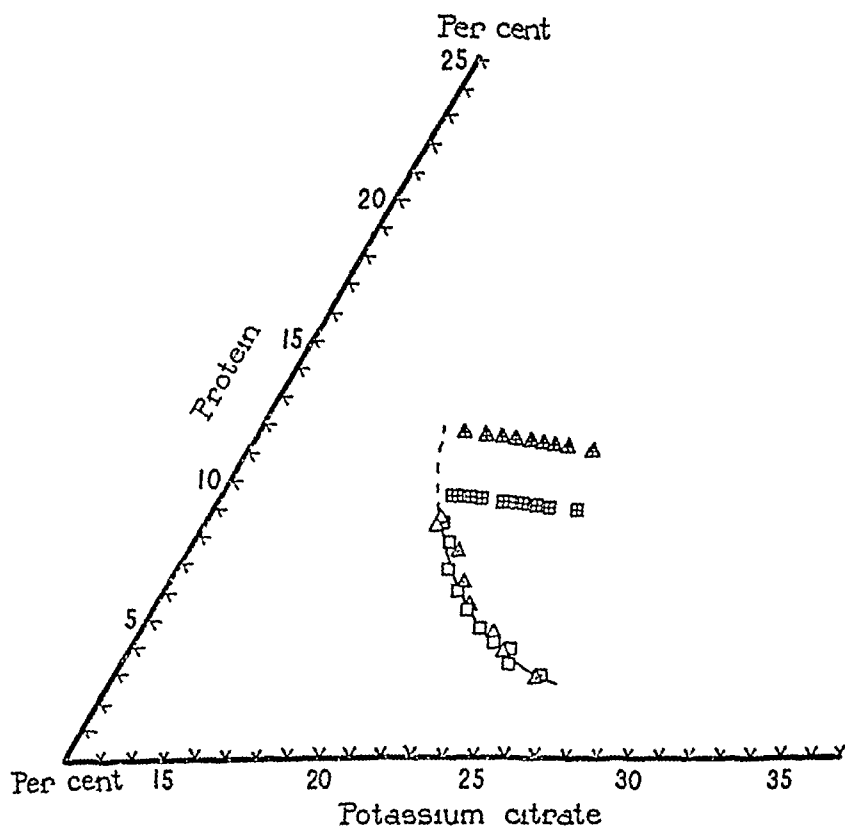


FIG 13 Horse serum

- = total composition, concentrate III  
 □ = liquid phase, " "  
 ▲ = total composition, " IV  
 △ = liquid phase, " "

I and II coincide at about 17.5 per cent citrate when both solutions are saturated with respect to the third globulin fraction. This fraction begins to separate at a lower salt concentration from the more concentrated protein solution. The curves of III and IV nearly coincide. As this fraction is separated as a crystalline substance it

was more difficult to get the solubility curve before the fractions decomposed. Although the coincidence of the lines is not perfect there is no evidence of fractionation. This fraction shows the characteristics of a single homogeneous protein.

#### DISCUSSION

The method described here is different from those used previously, in that the globulin has not been removed from its original medium and has been in so far as is possible retained in its original state. It may be that albumin or other substances normally present in the serum are necessary to prevent changes in the aggregation of the globulins. Only 3 to 4 days elapsed before all the precipitations were made, since the processes of purification are eliminated. A temperature of 0°C. was maintained thus retarding denaturation. The pH is within the stable zone.

The curves in Figs. 9, 10, 12, and 13 are those which were predicted by applying the phase rule to systems of several simple protein components.

Then, in all probability, the principal globulin that precipitated between 19 per cent and 24 per cent potassium citrate from calf serum is a homogeneous substance (either a single substance or a solid solution), a dispersion of particles of the same size, or an equilibrium system if made up of unit particles. It could not be a dispersion of particles of different sizes by a stabilizing agent, nor a compound of varying composition such as  $m \times n$ . In each of the latter cases there should not have been a parallelism between the two curves plotted in Fig. 11 nor a coincidence of the curves in Figs. 9, 10, 12, and 13 unless indeed the fractions which might make up the protein had practically the same solubility, which is unlikely. With, for example, two component parts of different solubilities we should have obtained a greater concentration of the more insoluble part in the higher concentrate (II) in Fig. 11. Hence one of two different solubility curves would have been found, either a change in direction at about 21 per cent, if a series of dispersed particles of varying sizes constituted the protein "B," or a gradual divergence throughout the curve from 19 per cent to 24 per cent citrate if B were made up of a varying complex of  $m \times n$ .



It does not necessarily follow that other fractions of serum are homogeneous because this fraction has been found to be so. In fact, there are indications that this fraction may be of a simpler nature than some of the others occurring in serum.

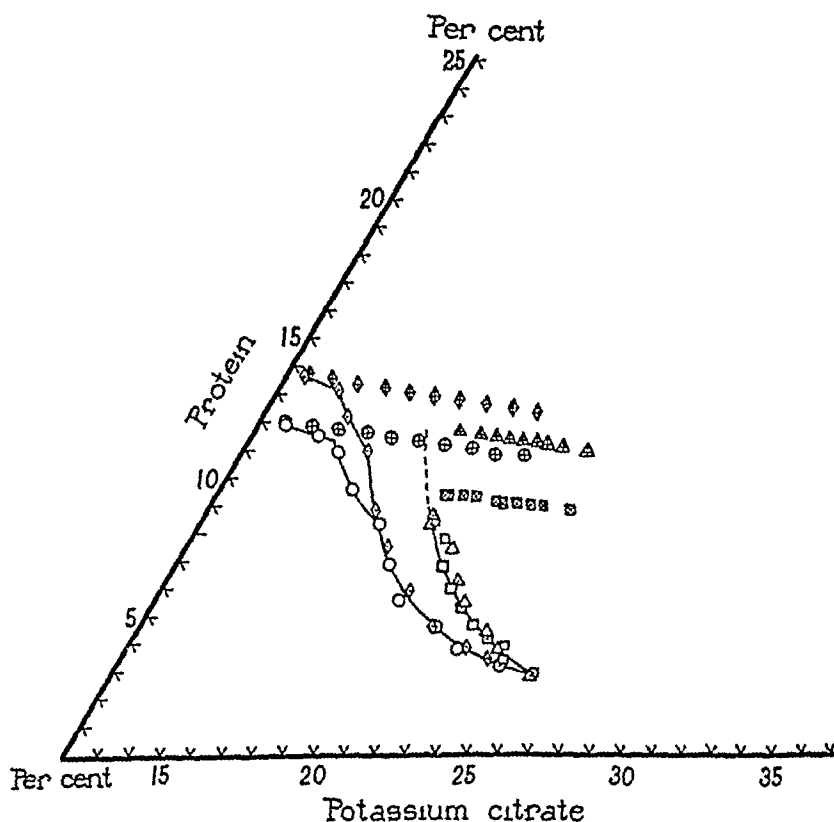


FIG 14 Horse serum Combination of Figs 12 and 13

- ⊕ = total composition, concentrate I
- = liquid phase, " "
- ⊕ = total composition, " II
- ◇ = liquid phase, " "
- ⊗ = total composition, " III
- = liquid phase, " "
- △ = total composition, " IV
- △ = liquid phase, " "

The case for a solid solution becomes very strong when the four curves in Fig 14 are compared. The four curves do not coincide until the potassium citrate becomes about 26 per cent although the two

curves in each pair converge during the separation of the fraction from 17 per cent to 26 per cent salt. It may be that we have a *solidus liquidus* curve with a minimum at 26 per cent salt when the descending salt concentration is plotted against the composition at a constant temperature. In such a case the curves in Fig 12 fall on one arm while those in Fig 13 fall on the other.

Since a solid solution is a homogeneous substance we seem justified in calling the principal globulin fractions, which have been considered in this paper, homogeneous.

#### CONCLUSIONS

A method has been developed for applying the phase rule to systems of several protein components in serum.

The globulin fractions which have been investigated appear to be homogeneous substances.

Throughout the duration of this borderline study of the application of the phase rule to a physiological problem, I have been indebted to Dr T Addis and Dr J W McBain for helpful criticism.

The calves for these experiments were kindly furnished by Mr H H Sortor of Tulare, California.

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# AREA AND VISUAL THRESHOLD

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(Accepted for publication, September 2, 1937)

## I

### INTRODUCTION

Increase in the area of a visual field results in orderly decreases in the threshold for light and color, the minimum separable and discriminable, the fusion interval,<sup>1</sup> and the latent period of electrical response in the retina, optic nerve, and cortex. Various of these relations have been observed in the conger eel, frog, rabbit, cat, and man.<sup>2</sup> The variation of each of the properties cited with area may be represented graphically by a smooth curve convex to the origin. In every case the effect of increase in area is an improvement in visual performance.

It is apparent that these phenomena reflect some basic retinal characteristic. Recently this has been assumed to be the reflex interaction of dispersed portions of the retinal surface. Various area relations have been attributed to summation, inhibition, and in one significant case to inhibition of summation (Graham and Granit, 1931).

If it were true that just proximal to the layer of rods and cones the impulses from widely separated retinal areas converge on common paths (Granit, 1932), then the exquisite image-forming mechanism

<sup>1</sup> By this term is meant the reciprocal of the minimum frequency at which an intermittent stimulus produces a 'fused' sensation.

<sup>2</sup> Investigations upon the visual threshold are referred to in the course of the paper. Work upon other functions includes minimum separable (Wertheim, 1894), minimum discriminable (Aubert, 1865, p. 86; Cobb and Moss, 1927), fusion interval (Granit and Harper, 1930), latent period of the electrical response in the retina and optic nerve of the conger eel and frog (Adrian and Matthews, 1927), in the retina of the cat (Granit, 1933), in the cortex of the rabbit (Bartley, 1935).

nisms of the eye, the correspondence of dimensions of retinal receptors with the optimal visual acuity (Helmholtz, 1911), and the relatively intact spatial projection of the retina upon the cerebral cortex (Lashley, 1934) should be alike meaningless. All these relations indicate a mosaic of relatively independent receptor-nerve units. It is the purpose of this paper to show that simple properties of a mosaic retina account qualitatively and quantitatively for the characteristic variation with field area of the visual threshold.

## II

*Measurements*

The threshold-area measurements reported in this paper were obtained in collaboration with Dr. Charles Haig at the close of an inves-

TABLE I  
(Effective Distance, Eye to Field, 385 mm.)

Field diameter	Angular diameter	Area	Relative area
<i>mm</i>		<i>sq mm</i>	
1 184	55 25'	30 06	1 00
2 421	1°53'	125 8	4 18
3 633	2°49'	283 2	9 42
4 932	3°50'	521 6	17 35
6 367	4°56'	869 6	28 93

tigation of dark adaptation in various retinal areas (Hecht, Haig, and Wald, 1935-36). The same apparatus was used, and indeed the experiments shown in Figs. 1 and 2 formed part of the latter research. I am greatly indebted to Professor Hecht and Dr. Haig for permission to use these data.

All of the original measurements reported have been performed upon the author's right eye. The dark adaptation procedure has already been described (Hecht, Haig, and Wald, 1935-36). In examining the effect of area on threshold, the subject was first dark adapted for 30 minutes, and then exposed to circular fields of various dimensions, fixated by means of a small bright "star" placed at various distances above the fields. The threshold of each field was determined three times consecutively. Definition of the outlines of the field was not required, the threshold response was the simple luminal light sensation. The field could be exposed at will by raising a blind, and was opened for successive flashes of

about 1 second duration in the course of a measurement. At times, at the end of a series of readings, initial measurements were repeated to find whether changes had occurred during the experiment. No significant change was ever detected.

Five fields were used, varying in relative area from 1 to 28.9, and in angular diameter from approximately  $1^\circ$  to  $5^\circ$ . These approximate visual angles are used to characterize the fields throughout the present paper. Their accurate dimensions are shown in Table I.

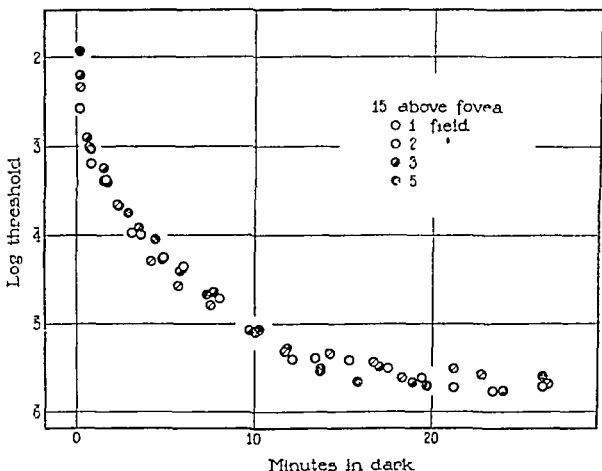


FIG. 1. Dark adaptation in fields fixated 15° above the fovea. The thresholds for the  $5^\circ$  field are in millilamberts. The remaining curves have been uniformly displaced on the *log threshold* axis to emphasize their identity in form.

Hecht, Haig, and Wald have shown that in centrally fixated fields wider than  $1^\circ$ , the fall in threshold of the dark adapted eye with increase in area is due primarily not to the change in area itself, but to variation in the rod-cone composition of the fields. This heterogeneity of the central retina is reflected in an orderly way in large changes in the form of the dark adaptation curves. The latter, therefore, offer a convenient index of variations in the character of the retinal population.



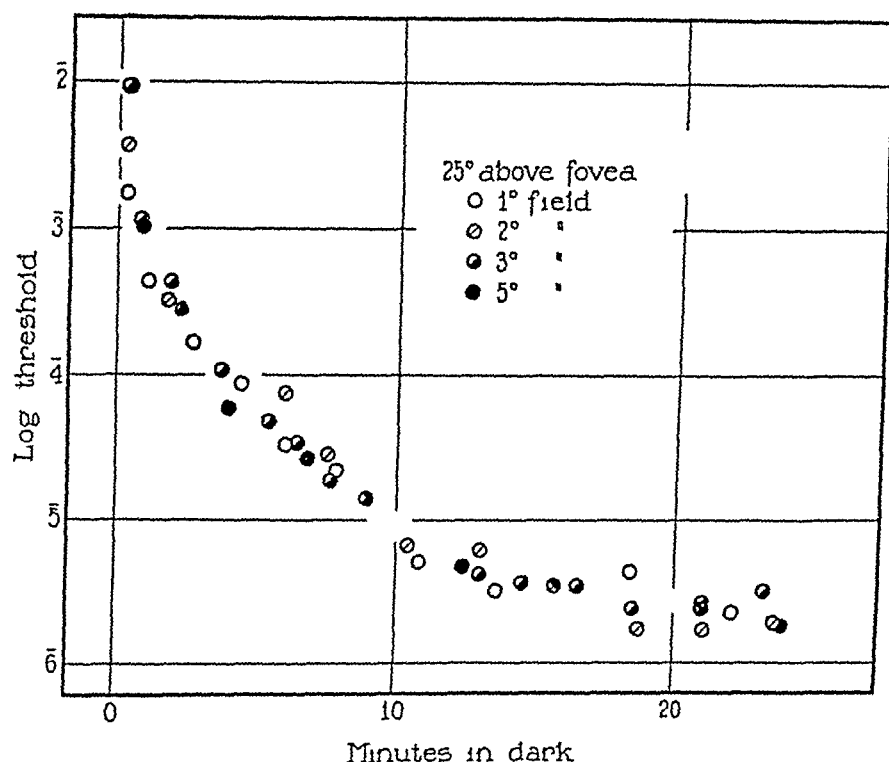


FIG 2 Dark adaptation in fields fixated 25° above the fovea Thresholds as in Fig 1

TABLE II

(1) Number of measurements	(2) Area	(3) Average log threshold	(4) Computed log threshold
15° above fovea			
	<i>sq cm</i>	<i>ml</i>	<i>ml</i>
9	0 301	$\bar{6}$ 82	$\bar{6}$ 81
9	1 258	$\bar{6}$ 24	$\bar{6}$ 28
12	2 832	$\bar{6}$ 14	$\bar{6}$ 14
9	5 216	$\bar{6}$ 06	$\bar{6}$ 05
9	8 696	$\bar{7}$ 97	$\bar{7}$ 97
25° above fovea			
6	0 301	$\bar{6}$ 99	$\bar{6}$ 98
8	1 258	$\bar{6}$ 43	$\bar{6}$ 43
10	2 832	$\bar{6}$ 16	$\bar{6}$ 21
7	5 216	$\bar{6}$ 03	$\bar{6}$ 06
7	8 696	$\bar{7}$ 98	$\bar{7}$ 94

In fields fixated  $15^\circ$  or  $25^\circ$  above the fovea, the shape of the dark adaptation curves remains unaltered as the field diameter is increased from  $1^\circ$  to  $5^\circ$  (Figs 1 and 2). Apparently within these limits the elementary composition of the fields remains constant.

In these homogeneous regions of the retina pronounced effects of area on threshold are found. At  $15^\circ$  above the fovea, increase in the field diameter from  $1^\circ$  to  $5^\circ$  lowers the threshold sevenfold, at  $25^\circ$  above the fovea, tenfold (Figs 3 and 4). The figures show the individual measurements. Averages of these data are presented in Table II. No attempt has been made to indicate in the figures several instances in which identical threshold readings were obtained repeatedly, therefore they show the range, but not always the precise weight of the measurements.

### III

#### *Analysis*

*General Considerations*—In any region of the retina the receptor units—rods, cones, or summing clumps of rods or cones—form a population within which retinal properties are distributed in various ways. Obviously the number of elements which possess a specific value of a property increases with the size of the field. If the population is homogeneous throughout the regions examined, this increase is proportional, and curves describing the distribution of the property among the elements are multiples of one another, proportional in height to the field area. This situation is presented schematically in Fig 5, in which for simplicity a linear form of distribution of a retinal property,  $x$ , is shown for a series of areas.

Most types of visual measurement appear to involve not the entire population of the retinal field, but a comparatively small number of units which are peculiarly susceptible to the stimulus. Tentatively one may assume that a threshold response involves the activity of a fixed number of retinal elements. Such an assumption appears in Fig 5 as a line drawn parallel to the abscissae. It cuts the distribution curves for increasing areas in decreasing values of the retinal variable,  $x$ . When values of  $x$  and area obtained from such a diagram are plotted graphically, they assume precisely the general form of all the area relations.

Several arbitrary steps which have appeared in this procedure are not essential. Any type of distribution of  $x$  among the elements does as well qualitatively as the linear form used in the diagram. Moreover, the distributions may vary in form or increase disproportionately with area. Even the assumption of the participation of a constant number of elements in the measurements is unnecessary.

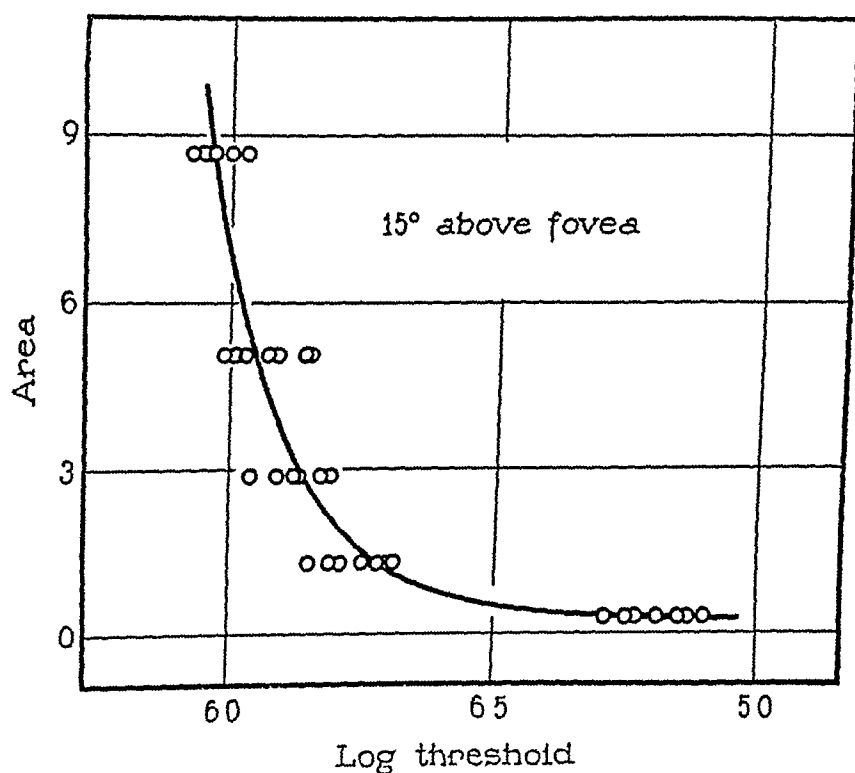


FIG 3 The area-threshold relation in fields fixated 15° above the fovea. Field areas in square centimeters, thresholds in millilamberts. The points are individual measurements taken from three experiments. The curve is theoretical, its equation is  $0.33 \log (A - 0.275) + \log I = -5.72$ .

The qualitative decrease in  $x$  with increase in area follows from any law included between this one and the assumption that the number of elements entering the measurements is proportional to the area of the field, which would appear in the diagram as a line parallel to the ordinates. It is shown below that actually the threshold number of active elements, if it increases at all, does so much more slowly than the field area.

Ultimately, only the basic concept of the retina as a population of relatively independent units is indispensable to the analysis. With this accepted, the characteristic type of dependence of all retinal properties upon area follows inevitably.

*Area and Threshold*—Applied specifically to the area threshold problem, these considerations reduce to two propositions, the first implicit in the homogeneity of the retinal areas examined, the second a reasonable assumption concerning the threshold.

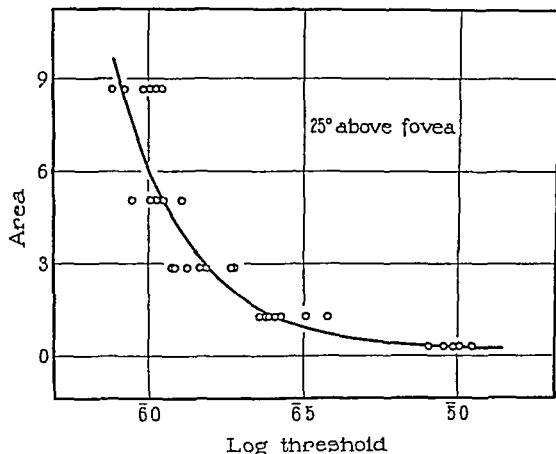


FIG. 4. The threshold area relation in fields 25° above the fovea. Units as in Fig. 3. The points are from two experiments. The curve is theoretical; its equation is  $0.54 \log (A - 0.20) + \log I = -5.56$ .

1. Throughout all portions of a homogeneous retinal region the percentage of elements which possess a specific intensity threshold is the same. In a series of fields of various sizes located within such a region, the number of elements of fixed threshold is directly proportional to the field area. The integral distribution curves for such fields are therefore simple multiples of one another. This is the situation shown in Fig. 5 and obtaining in the present experiments.

2 A clue to the significance of the threshold was furnished by a subjective observation noted consistently throughout these experiments. Thresholds were always measured to the liminal light sensation, regardless of definition of the field. Nevertheless, when this limen is attained in a  $1^\circ$  field, its boundaries are sharply defined. At the threshold of a  $2^\circ$  field, its boundaries have become hazy. This

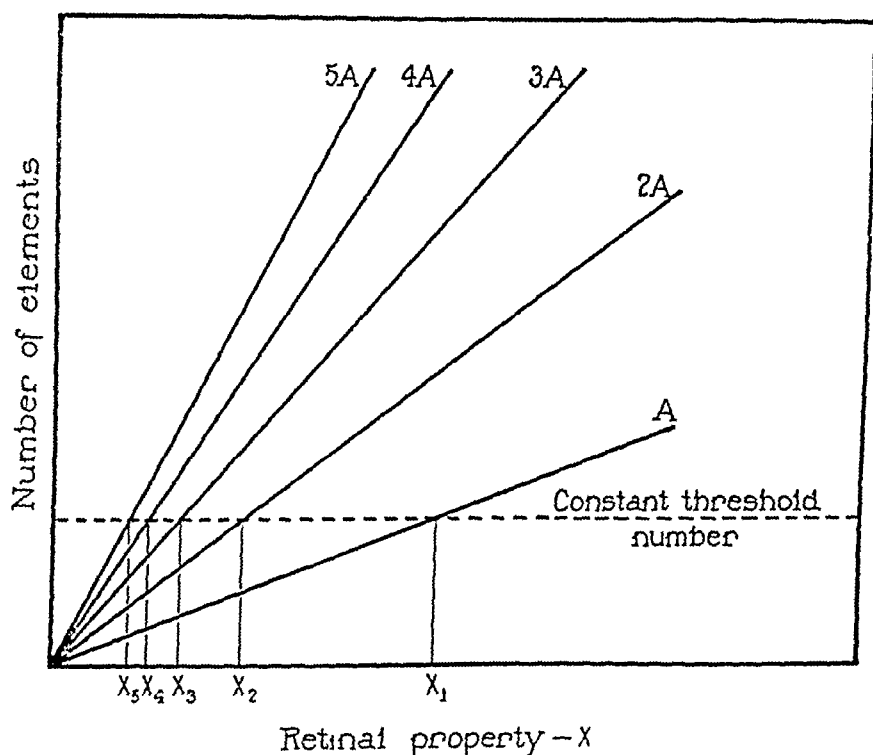


FIG. 5 The mechanism of variation of a retinal property,  $x$ , with field area. The heavy lines are hypothetical integral distributions of the property in a series of areas,  $A$  to  $5A$ . As the area increases, a constant threshold number of elements respond at decreasing values of the property,  $x_1$  to  $x_5$ .

dissipation of the sensation increases rapidly with further increase in area. Finally, in  $5^\circ$  fields no spatial impression at all remains, but merely a formless sensation of light flashing on and off as the blind is raised and let fall.

If the threshold corresponded with the excitation of a constant density of retinal elements, all fields should have been equally well defined. The actual responses are as though at the threshold a

number of elements is active, sufficient to define a small field but too small to resolve a large one. This number, if it increases at all, does so much more slowly than the area. We shall assume that it remains constant for all the fields *that the threshold corresponds to the stimulation of a constant number of elements*.

This assumption does not imply reflex interaction among the threshold elements. The formal conditions of the analysis are fulfilled if the threshold number is taken to be 1. Since in the present experiments the form of the smallest field is resolved, more than a single element must respond at these thresholds. But this very observation demonstrates that this is not a case of spatial summation, of convergence upon a final common path. For a summing group of elements must behave as a unit, and therefore cannot resolve an image.<sup>3</sup> Certainly some integration of responses from the individual elements which enter the threshold is implied. That this is probably central is shown by experiments upon the binocular threshold, which most investigators have found to be lower than the monocular (Piper, 1903 *a*, Roelofs and Zeeman, 1914, Shaad, 1934). In forming the binocular threshold, impulses from the single retinas must reach the centres, yet fail to elicit a sensation. It seems reasonable to suppose that these subliminal reactions consist of impulses from fewer than the constant threshold number of elements.<sup>4</sup>

*Derivation of the Threshold Distribution*—Though any type of function for the distribution of thresholds yields the correct qualitative area threshold relation, for a quantitative description of the phenomenon a "true" distribution is needed. With the use of the constant threshold number assumption the requisite portions of this may be derived directly from the data.

The following procedure yields the threshold distribution in the 1°

<sup>3</sup> König (1897) has shown that at the threshold of a large unfixated field the human eye can resolve a minimum separable of about 25 minutes  $\pm$ , that active elements are about 0.11 mm apart on the retinal surface.

<sup>4</sup> I believe Beitel's experiments (1934) to provide another example of this phenomenon. Beitel found that two adjacent subliminal test patches may evoke the liminal response when applied simultaneously. He concluded this to be an example of spatial summation. I should prefer to assume that each patch stimulated fewer than the threshold number of elements, both together just this number.

field,  $25^\circ$  above the fovea At  $\log I = \bar{6} 99$ , the constant threshold number of elements,  $n_t$ , is active in this field of relative area, 1 00 At  $\log I = \bar{6} 43$ ,  $n_t$  elements operate in the  $2^\circ$  field of relative area, 4 18, therefore at this intensity  $n_t/4 18$  elements are active in the  $1^\circ$  field At  $\log I = \bar{6} 16$ ,  $n_t$  elements are stimulated in the  $3^\circ$  field of relative area, 9 42, therefore at the same intensity  $n_t/9 42$  elements

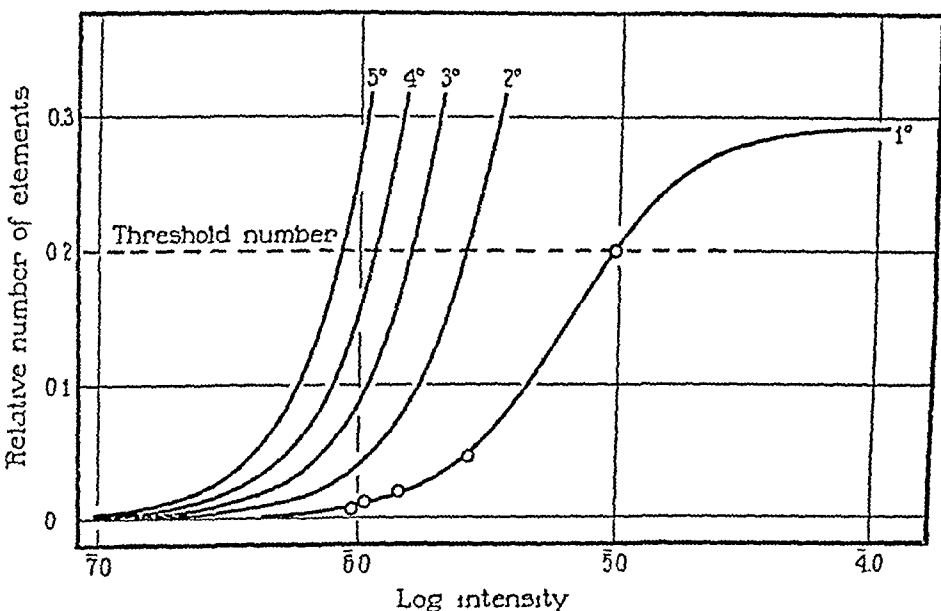


FIG 6 Graphic analysis of the threshold-area relation,  $25^\circ$  above the fovea The open circles were calculated from the averaged data of Table II as described in the text The distribution curves conform to the equation,  $0.54 \log \left( \frac{A}{n} - 1 \right) + \log I = -5.20$  The constant threshold number line cuts them in theoretical thresholds for the various areas

are active in the  $1^\circ$  field By completing this process five points are obtained on the distribution curve which relates the illumination to the relative number of active elements in the  $1^\circ$  field The distributions in the larger fields are simple multiples of this, proportional to area

The points obtained in this way for the  $1^\circ$  field,  $25^\circ$  above the fovea, are plotted in Fig 6 The curve drawn through them is

theoretical, and is described below. The data for fields  $15^\circ$  above the fovea yield similar results. It is apparent that the points themselves adequately complete a quantitative analysis. This may be recapitulated: (1) Using the constant threshold number assumption, the relevant portion of the threshold distribution in one area is obtained from the area threshold data. (2) The distributions for other areas are simple multiples of this. (3) The intercepts of these distributions with the constant threshold number line at  $n_t$  regenerate the original data.

*Derivation of an Area Equation*—A general equation for the type of distribution derived in the preceding section would be very useful, since from it a rational formula for the area threshold relation might be obtained. The points derived in the previous section describe correctly the beginnings of the distribution function. Obviously it must end by becoming parallel to the abscissa, when the total number of elements in the area becomes active. The complete function is, therefore, sigmoid in form, when threshold is plotted logarithmically. Hecht (1928-29) has concluded from quite independent evidence that the distribution of log threshold intensity is sigmoid.

A number of formulae for sigmoid curves have been applied in the present analysis with varying degrees of success. All of them yield roughly the right type of answer. This is inevitable, for even so naive a diagram as Fig. 5 does so. The equation of the lines in Fig. 5 is  $n = kAI$ , in which  $n$  is the number of active elements,  $A$ , area,  $I = x$  = intensity and  $k$  is a proportionality constant. If  $n$  is held constant to the threshold value  $n_t$ , the equation becomes  $AI = n_t/k = \text{constant}$ . This relation is familiar as "Ricci's law" (1877), it is in exact but encouragingly close to a correct solution.

One expression has been peculiarly successful in describing the present and related data. I shall derive this by two methods with quite different, though not mutually exclusive, implications.

1. Hecht (1928-29) has based the form of the threshold distribution upon the photochemical steady state. The equation for this may be written (Hecht, 1934-35)

$$KI = \frac{x^p}{(a-x)^q}$$



in which  $I$  = intensity,  $x$  = concentration of photoproducts,  $a$  = maximum value of  $x$ , and  $K$ ,  $p$ , and  $q$  are constants. Rewritten in the form of a threshold distribution this becomes

$$KI = \frac{n^p}{(A - n)^q}, \text{ or}$$

$$(A - n)^q I = n^p / K,$$

in which  $A$ ,  $n$ , and  $I$  are respectively the field area, number of active elements (really the "active area"), and intensity. If  $n$  is held constant to the threshold value  $n_t$ , this reduces to

$$(A - n_t)^q I = n_t^p / K = \text{constant}, \quad (1)$$

the desired area-threshold relation

2 The second derivation assumes no mechanism, and possesses the advantage of being sufficiently general to be applied to other area phenomena in addition to the threshold. It is based upon Verhulst's so called logistic formula (1838), a symmetrical sigmoid function. This is of the form

$$y = \frac{K}{1 + Ce^{-mx}}$$

in which  $y$  and  $x$  are variables,  $K$  is the maximal value of  $y$ , and  $C$  and  $m$  are constants. As a threshold distribution this may be written

$$(a) \quad n = \frac{A}{1 + Ce^{-m \log I}}$$

This is exactly equivalent to Hecht's photochemical steady state equation for the case  $p = q = 1/0.4343m$

$$(b) \quad nCe^{-m \log I} = A - n$$

$$(c) \quad \log nC - 0.4343m \log I = \log (A - n)$$

If  $1/0.4343m$  is set equal to a new constant,  $k$ , this becomes

$$k \log (A - n) + \log I = k \log nC \quad (2)$$

When  $n$  is held constant to the threshold value  $n_t$ ,

$$k \log (A - n_t) + \log I = k \log n_t C = \text{constant}, \quad (3)$$

or, removing the logarithmic notation,

$$(A - n_i)^k I = \text{constant.} \quad (4)$$

This is an area threshold equation identical with (1). Written as in (3) it is the equation of a straight line of slope  $k$  and intercept  $k \log n_i C$ . In this form it may readily be tested with the data.

With  $A$  expressed in square centimeters, if  $n_i$  is set equal to 0.275 for fields  $15^\circ$ , and 0.20 for those  $25^\circ$  above the fovea, the straight lines of Fig. 7 result. Their slopes and intercepts, substituted in equations (3) and (4), yield the following completed area threshold equations

$$\begin{aligned} 15^\circ \text{ above fovea} & \begin{cases} 0.33 \log (A - 0.275) + \log I = -5.72 \\ \text{or} \\ (A - 0.275)^{0.33} I = 1.9 \times 10^{-4} \end{cases} \\ 25^\circ \text{ above fovea} & \begin{cases} 0.54 \log (A - 0.20) + \log I = -5.56 \\ \text{or} \\ (A - 0.20)^{0.54} I = 2.75 \times 10^{-4} \end{cases} \end{aligned} \quad (5)$$

From these equations the curves of Figs. 3 and 4 have been computed. It is clear that they describe correctly the course of the individual measurements. The equations have been used also to compute theoretical values of *log threshold* for the areas investigated. These are shown in column 4 of Table II, they agree very closely with the experimental averages.

By substituting the appropriate values of  $k$  and  $C$  into equation (2), and allowing  $n$  to vary while  $A$  is held constant, the theoretical threshold distributions may be computed. In this way the distribution curves of Fig. 6 were obtained. The curve for the  $1^\circ$  field fits the points derived by direct analysis of the data. The series of curves is cut by the appropriate constant threshold number line in theoretical values of *log threshold*, corresponding with those computed arithmetically from the equations and shown in Table II.

Piper (1903 b) first proposed for peripheral fields the empirical expression,  $\sqrt{A} I = \text{constant}$ . Henus (1909) and Fujita (1909), working in the same laboratory, showed this to hold roughly in fields  $1^\circ$  to  $10^\circ$  in diameter, and beyond these limits not at all. An equivalent expression, proposing reciprocity of field diameter and threshold,

has been shown by Piéron (1920 *a, b*) to hold in neither small peripheral nor foveal fields

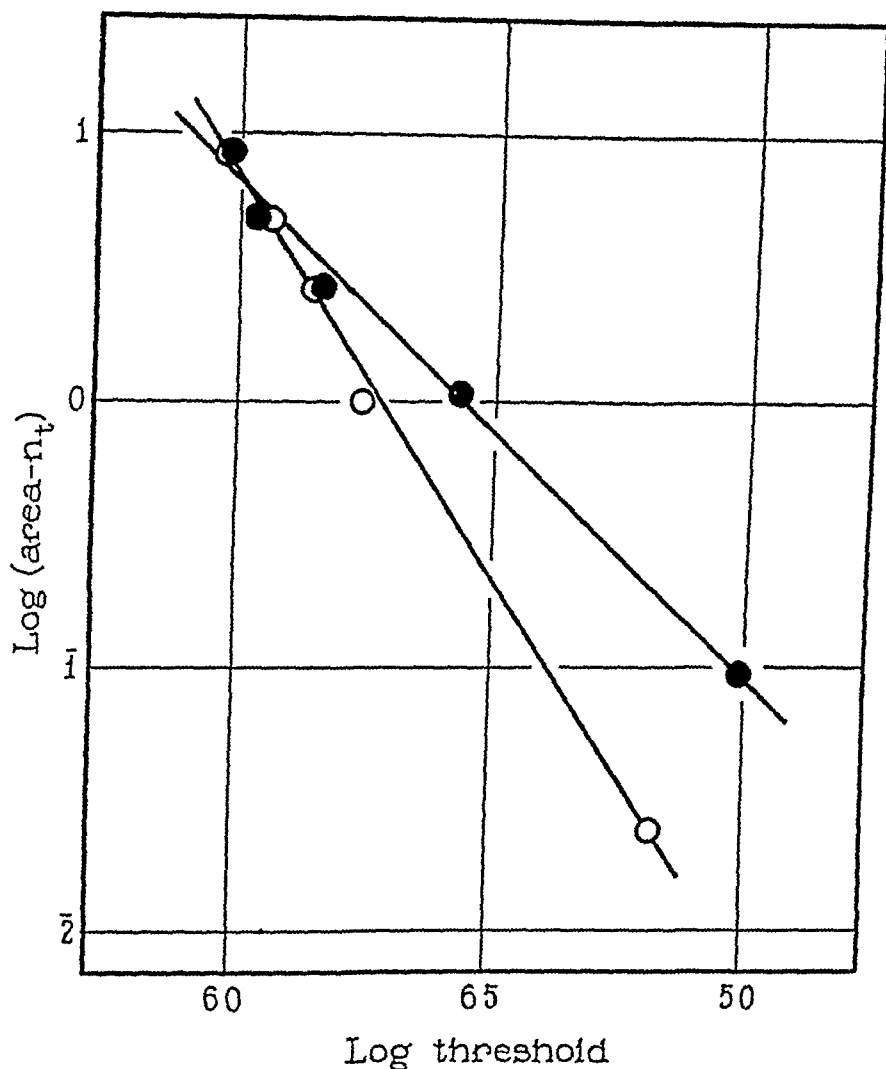


FIG 7 Linear graph of the threshold-area relation, 15° (open circles) and 25° (closed circles) above the fovea. Areas in square centimeters, thresholds in millilamberts

The present analysis has led to the general expression  $(A - n_t)^k$   $I = \text{constant}$ . When the threshold number of elements,  $n_t$ , is a small fraction of the total number in the visual field,  $A$ , the term  $(A - n_t)$  approaches  $A$  as its limiting value. This condition is fulfilled in

quite small foveal fields, in which the concentration of receptor units—presumably individual cones—is very high. It should obtain only in much larger peripheral fields, since in the periphery the retinal elements are comparatively large summing clumps of rods. In both cases the threshold area equation reduces to the simple form,  $A^k I = \text{constant}$ <sup>5</sup>

If, with  $n$ , a small fraction of  $A$ ,  $k$  chance to take the value 0.5, the equation becomes  $\sqrt{A} I = \text{constant}$ , or Piper's rule. This is a rough approximation to the present solution, in which  $k$  is found to equal 0.33 and 0.54 in two peripheral regions.

#### IV

##### *Foveal (Cone) Thresholds*

A number of investigators have measured the area threshold relation in small fields, centrally fixated. When the diameters of the fields do not exceed 1.5°, they may be assumed to fall wholly within the fovea and to stimulate a relatively homogeneous population of cones. The analysis proposed here should be directly applicable to such data.

Experiments of Abney (1897) and Piéron (1920 *b*) have been examined. In both cases the direct plot of *log area* against *log threshold* yields straight lines. This relation was discovered empirically by Abney. Apparently the threshold number of elements,  $n_t$ , is a negligible fraction of the total populations of these fields.

The data of Abney obtained with test illuminations of wave length 527 mμ are shown in Fig. 8. Their equation is  $0.85 \log A + \log I = 0.49$ . Data obtained at other wave lengths are described by similar expressions, differing only in the terminal constant. Piéron's data agree reasonably well with the equation,  $0.88 \log A + \log I = -4.37$ . The variation in terminal constants here is due to the use of different units of area and intensity. The slope constants are independent

<sup>5</sup> This expression was discovered empirically by Abney (1897). Its limitations in small fields (under 1.8 diameter) have been defined by Abney and Watson (1916) within the fovea or in monochromatic red light—both conditions for stimulating cones—the rule holds in fields of other colors outside the fovea it fails. These restrictions agree sensibly with our theoretical expectations.

of these units and agree well with each other Both equations may be combined in the form  $A^{0.85-0.88}I = \text{constant}$

Riccò (1877) and again Charpentier (1882) proposed for foveal fields the empirical rule,  $AI = \text{constant}$  This expression states that the liminal foveal response is evoked by a constant flux of light, regardless of its spatial dispersion The simplicity of this rule has preserved it in spite of repeated demonstrations of its failure to describe accurate data (Aubert, 1865, Abney, 1897, Abney and Wat-

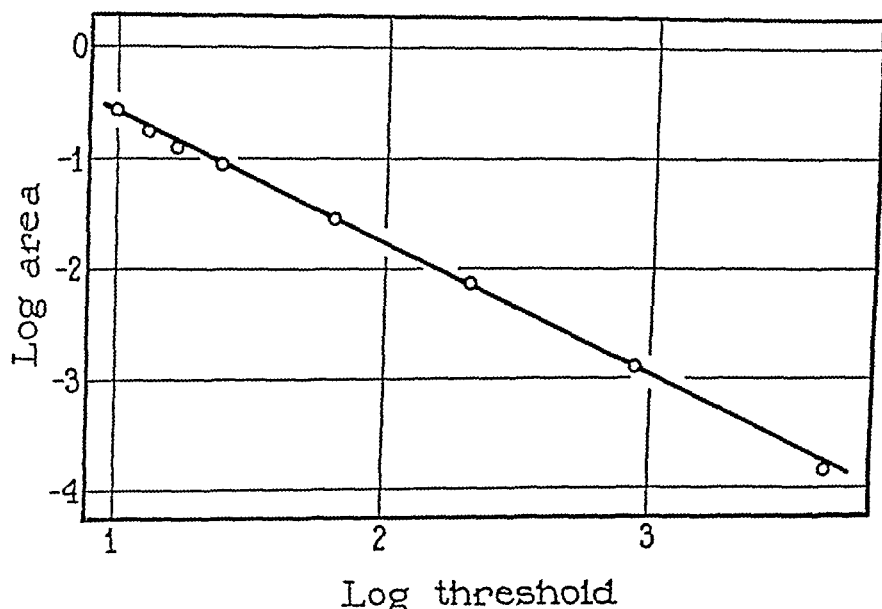


FIG 8 Foveal thresholds to light of wave length 527 mμ, as a function of field area Data of Abney (1897)

son, 1916, Piéron, 1920 b) "Riccò's law" is still widely accepted as a basis for theoretical discussion

With  $n_i$  a small fraction of  $A$ , if  $k$  chance to assume the value 1, the general expression  $(A - n_i)^k I = \text{constant}$  reduces to  $AI = \text{constant}$ , or Riccò's law The accurate values of  $k$  in the two sets of data we have analyzed are 0.85 and 0.88 In other cases they may fall below 0.5 (data of Watson, in Abney and Watson, 1916) No basis exists, therefore, for the acceptance either of Riccò's rule or of its implication of perfect spatial summation in the fovea (Creed, 1931, Houstoun, 1932)

## DISCUSSION

The quantitative development of the present area threshold analysis rests upon the assumption that at the threshold a constant number of elements respond. This is probably approximately true for a wide range of areas. It may be expected to fail both in very small and very large fields.

Very small fields require fairly intense light to stimulate them. This probably excites certain of their elements to respond at well above their liminal frequencies. The threshold in such cases is likely to correspond more nearly to a constant over all frequency of response than to a constant number of active elements. The threshold number of elements may be expected to decrease, as the frequency of response of individual elements rises. In the extreme case, it is likely that any single element, responding at sufficiently high frequencies, may excite the liminal sensation.

A more fundamental consideration, however, is the fact that the present analysis as a whole applies only to populations of elements sufficiently large to permit statistical treatment. It states nothing, therefore, about the behavior either of single elements or of very small fields.

In very large fields the threshold number of elements may be expected eventually to rise, due to the difficulty of distinguishing a very low density of active elements against the persistent background of visual "*Eigenlicht*".

The quantitative treatment, therefore, rests admittedly upon a reasonable approximation. It yields the threshold area equation,  $(A - n_i)^k I = \text{constant}$ , which describes the available data accurately, and is the general form of previous empirical formulae for this function. Whether the quantitative theory is finally to be regarded as entirely rational, or as a rational approach to a correct empirical solution, is of little present importance.

Throughout this investigation area phenomena have been assumed to originate in peripheral structures. Since the mosaic character of the retina appears to be transmitted relatively intact as far as the occipital cortex (Lashley, 1934), this point of view is unimportant. It is probable that area relations amenable to the present type of analysis occur at all levels of the visual pathways.

## SUMMARY

1 The variation of threshold with field area was measured in fields homogeneous in rod-cone composition. At  $15^\circ$  above the fovea, an increase in field diameter from  $1^\circ$  to  $5^\circ$  reduces the threshold sevenfold, at  $25^\circ$  above the fovea tenfold.

2 These changes are shown to follow qualitatively from simple statistical properties of the retinal mosaic. Analytic treatment leads to the expression,  $(A - n_i)^k I = C$ , in which  $A$  = area,  $n_i$  = constant threshold number of elements,  $I$  = threshold intensity, and  $k$  and  $C$  are constants. This equation describes the available data accurately, and is the general form of previous empirical area-threshold formulae.

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## STUDIES ON ENZYMATIC HISTOCHEMISTRY

### XXV A MICRO METHOD FOR THE DETERMINATION OF CHOLINE ESTERASE AND THE ACTIVITY-PH RELATIONSHIP OF THIS ENZYME\*

By DAVID GLICK\*\*

*(From the Carlsberg Laboratory Copenhagen)*

*(Accepted for publication, October 21, 1937)*

Within recent years a great deal of interest has been aroused concerning the enzyme hydrolyzing choline esters because of its relation to the metabolism of acetyl choline. Up to the present time there has been no adequate quantitative method for the determination of this enzyme in minute amounts of material, a determination particularly valuable for investigations of the enzyme in microtome sections of fresh frozen tissue for correlation of the activity with the histological structure of the tissue in question. The procedure to be described was based on the principle of the micro method developed for estimation of esterases hydrolyzing esters of simple alcohols (1). The sensitivity of the method is such that splitting can be measured down to the equivalent of the liberation of 0.20 c mm N/20 acid, corresponding to  $1 \times 10^{-8}$  mol of ester, or 1.81% of acetyl choline chloride.

The pH range within which digestions may proceed in the method employed is limited, hence it is necessary to ascertain beforehand whether this range of pH coincides with the region of maximum activity of the enzyme. Previously it had been shown that maximum activity in human serum occurred at a pH of 8.4-8.5 (2). In order to determine whether this optimum holds as well for the enzyme from different sources, and also because of the fact that the data would

\* This paper also belongs to the series of investigations by the author and coworkers as 'Studies in histochemistry XIII,' the preceding papers of which have appeared chiefly in the *Journal of Biological Chemistry*.

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be of value in other connections, studies of the activity-pH relationships were undertaken for the enzyme from horse serum, gastric mucosa of the pig, and cat brain

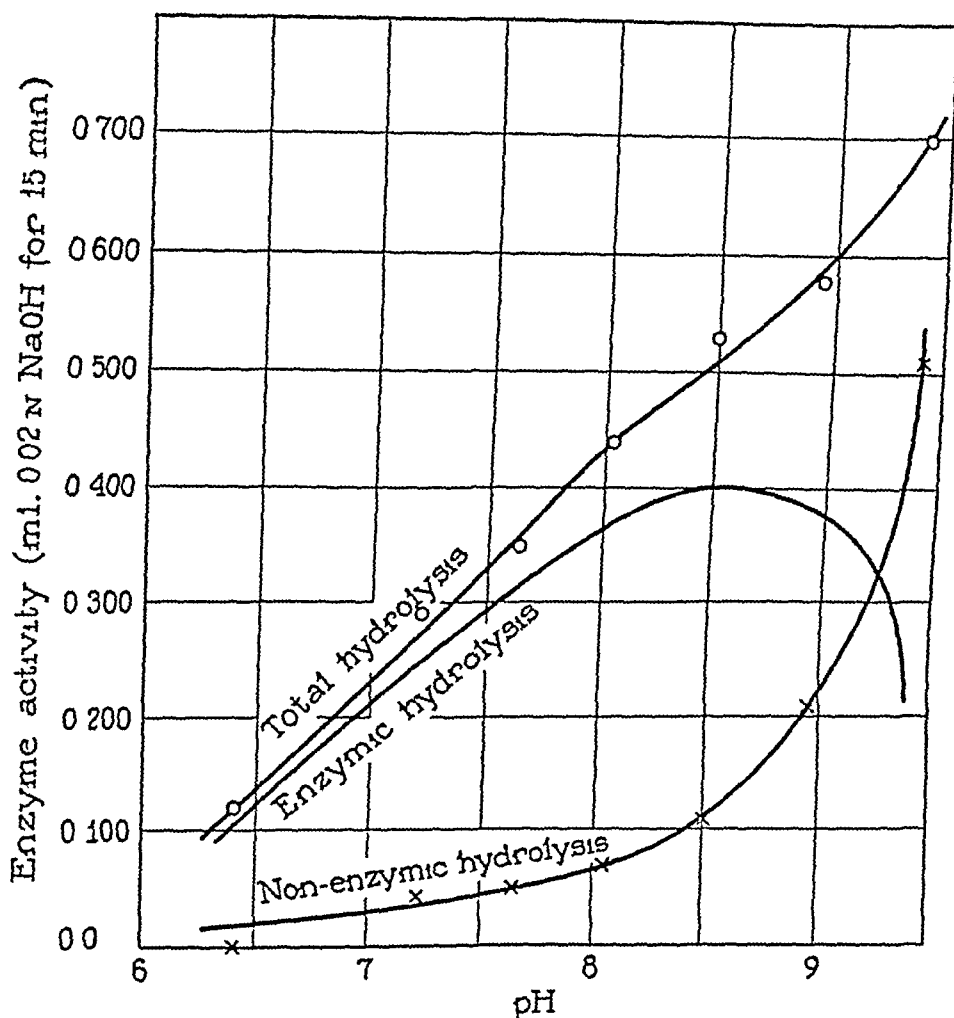


FIG 1 Activity-pH curve for choline esterase in pig's gastric mucosa

### *The Activity-pH Relationships*

The method used for the purpose of investigating the activity-pH relationships was the continuous electrometric titration procedure employing a glass electrode in the manner previously described (2). The advantages of this method, which eliminates the effects of buffer, indicators, etc., have already been discussed (2). For the study at hand, mechanical stirring was employed, and a burette graduated in 0.01 ml divisions was used for titration, the quantity of all alkali added

being measured on the burette rather than by counting the number of drops delivered. Measurements were made at 25° and the reaction mixture consisted of 20 ml of 0.2 per cent acetyl choline chloride plus 1 ml of enzyme preparation or 0.2 ml serum. In the case of dog stomach mucosa and cat brain the preparation was made by extracting 100 gm of finely ground tissue with 300 ml. of 30 per cent glycerol at 0° for 10 days, and subsequently filtering through paper. The results are given in Figs 1-3 and it may be seen that the optimum occurs at a pH of 8.5 in every case.

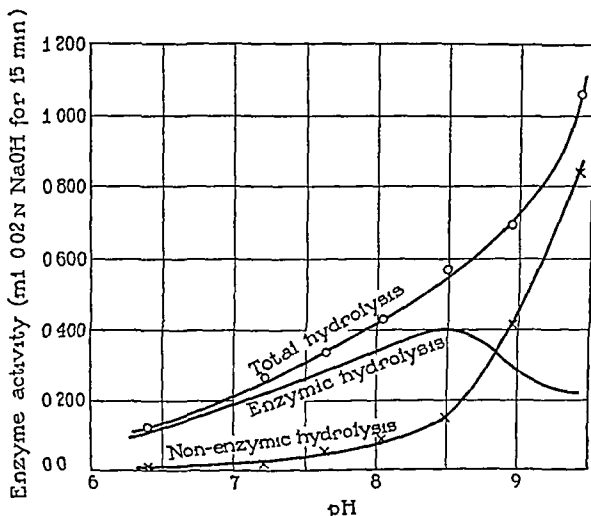


FIG 2 Activity pH curve for choline esterase in horse serum

### *The Micro Method for the Estimation of Choline Esterase*

In the micro method for determination of esterases splitting esters of simple alcohols to which reference has already been made a glycine NaOH buffer having a pH of 8.7 was employed, the enzyme activity was halted at the end of the reaction period by a phenol solution and the titration was finally carried out to an end point of pH 6.5 with brom thymol blue indicator.

From the curves in Figs 1-3 it is apparent that at the pH of optimum activity

the non-enzymatic hydrolysis begins to assume relatively large values. In order to minimize this effect without deviating greatly from the conditions for optimum activity, it was decided to employ a pH of 8.0. However, at this pH the glycine buffer approaches the lower limit of its buffering range, hence the veronal buffer of Michaelis (3) was used instead. The veronal buffer was prepared by adding 7.15 ml of 0.1 M sodium diethylbarbiturate to 2.85 ml of 0.1 M HCl.

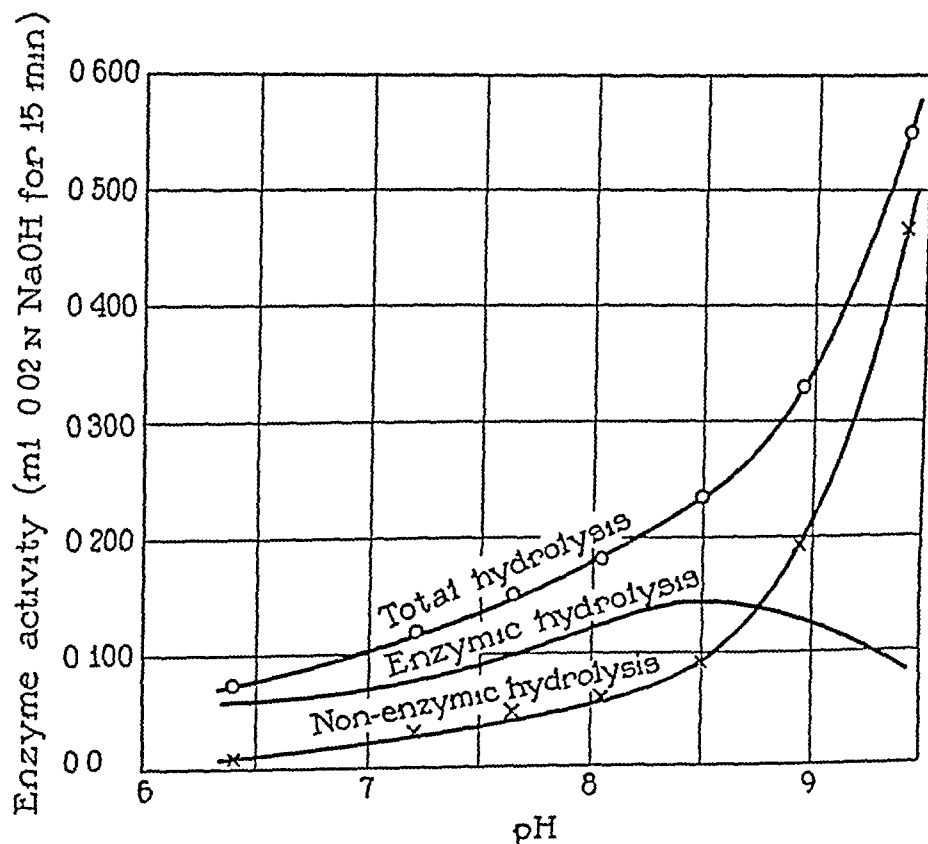


FIG. 3 Activity-pH curve for choline esterase in cat brain

Since the veronal has a lower  $pK$  (8.0) than the glycine buffer (9.7) it is necessary to carry the titration to an end point of lower pH in order to obtain a sharp color change. The lowering of the end point pH, however, is limited by the fact that the  $pK$  of the acetic acid liberated from the substrate is 4.7, and the pH of the end point must not be allowed to approach this value too closely or the sharpness of the color change will suffer. The pH finally chosen as most suitable for the end point was 6.2. As in the previous esterase

method (1), the standard color-comparison tube was filled with phosphate buffer and indicator, but the buffer was made up to a pH of 6.2 in this case

Because of its powerful inhibiting action upon choline esterase, eserine was used to stop the enzymatic reaction at the end of the digestion period. A solution of 10 ml of 0.1 per cent eserine sulfate

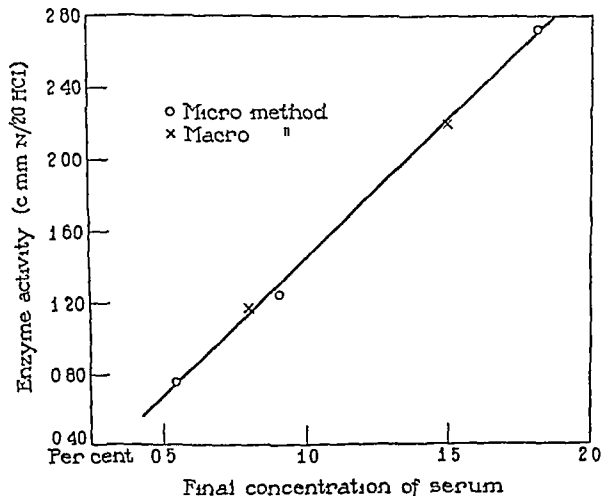


FIG. 4 Comparison of micro and macro methods

added to 1.5 ml of 0.04 per cent brom thymol blue was used for the purpose

The most desirable substrate concentration would be that giving maximum enzymatic hydrolysis with the least accompanying non-enzymatic splitting. In the case of choline esterase there exists for a given concentration of enzyme a concentration of substrate beyond which no significant increase in enzyme hydrolysis is demonstrable (2, 4). Therefore it is not only of no value to employ substrate concentra

tions greater than this quantity, but it is definitely objectionable since the magnitude of the non-enzymatic hydrolysis becomes greater, thus decreasing the accuracy of the measurement. For the present purposes a final concentration of about 0.4 per cent acetyl choline chloride was found to be entirely suitable.

TABLE I  
*Measurements of Choline Esterase Activity by the Micro Method*

Final serum concentration, <i>per cent</i>	0.55	0.91	1.82
N/20 HCl required, <i>c mm</i>	10.66	10.00	8.36
	10.70	9.98	8.36
	10.52	9.86	8.24
	10.58	9.90	8.44
	10.60	10.10	8.22
Average	10.61	9.97	8.32
Controls	11.44	11.30	11.10
	11.28	11.16	11.02
	11.40	11.20	11.00
Average	11.37	11.22	11.04
Difference	0.76	1.25	2.72

TABLE II  
*Measurements of Choline Esterase Activity by the Macro Method*

Final serum concentration, <i>per cent</i>	0.80	1.50
N/50 NaOH required, <i>ml</i>	2.98	4.96
Non-enzymatic hydrolysis	0.60	0.60
Difference	2.38	4.36
Expressed as <i>c mm</i> N/20 HCl for each 25.2 <i>c mm</i> of reaction mixture	1.19	2.20

Preliminary experiments with horse serum showed that practically the same activity was obtained in the presence of veronal as in glycine buffer. Furthermore the eserine solution employed was found to completely inhibit the enzymatic action.

The micro method described was finally compared with the macro electrometric procedure used for the activity-pH studies. This comparison should demonstrate

any effect of veronal buffer on the esterase activity. Accordingly a reaction mixture was employed for the micro titrations consisting of 16 c mm of 0.1 M veronal buffer at pH 8.0 containing  $\frac{1}{2}$  per cent acetyl choline chloride, plus 9.2 c.mm of horse serum solution. Digestion was allowed to proceed for 2 hours at 25°, after which 50 c mm of eserine indicator solution were added and titration carried out with N/20 HCl. Suitable control experiments were included in which the enzyme and substrate buffer (in the same tube) were not allowed to mix. Table I gives the results obtained.

The reaction mixture used in the macro experiments contained the same final substrate concentration. After addition of horse serum solution to the substrate the volume was made up to 20 ml with distilled water. After digestion for 2 hours at 25° and at a pH of 8.0 the quantity of N/50 NaOH consumed by the acid liberated is given in Table II.

The agreement between the two methods is apparent from Fig. 4. Hence it may be concluded that veronal has no measurable effect upon choline esterase under the conditions given.

#### SUMMARY

The activity-pH relationship for choline esterase from horse serum, gastric mucosa of the pig, and cat brain was investigated, and an optimum was observed at pH 8.5 in each case.

A micro method for the determination of choline esterase was developed capable of measuring the hydrolysis down to the order of that given by  $1 \times 10^{-8}$  mol of ester.

The author wishes to thank Professor S. P. L. Sørensen for his interest in this work, and to express his appreciation to Dr. K. Linderstrøm-Lang for his many helpful suggestions, and to G. Haugaard for the use of his glass electrode equipment.

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## STUDIES ON ENZYMATIC HISTOCHEMISTRY

### XXVI THE HISTOLOGICAL DISTRIBUTION OF CHOLINE ESTERASE IN THE GASTRIC MUCOSA NORMALLY AND AFTER ADMINISTRATION OF CERTAIN DRUGS\*

By DAVID GLICK\*\*

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*(Accepted for publication, October 21, 1937)*

In previous papers of this series Linderstrøm Lang, Holter, and coworkers have investigated the histological distribution of pepsin (1, 2), acid (2, 3), peptidase (2, 4), and esterase (2, 5) in the gastric mucosa of pigs, and the urease (6) in dog's stomach. The present investigation is a continuation of this work with regard to choline esterase. In view of the well known theory of chemical mediation of nerve impulses, choline esterase should be of particular interest in those organs, such as the stomach, which are influenced by the parasympathetic nervous system.

Dale and Feldberg (7) have demonstrated the liberation in the stomach of a substance indistinguishable from acetyl choline when the thoracic vagi were stimulated. These investigators pointed out the uncertainty regarding the point at which the acetyl choline is set free in the stomach. The possibility that it might be produced in the mucous membrane rather than in the muscle coats has been discounted by them since they observed large increases in output only when vagus stimulation threw the muscular wall into vigorous contraction. One would expect the region in which the acetyl choline is chiefly liberated to be the site of the greatest concentration of the enzyme hydrolyzing this substance, since choline esterase is an important factor in the physiological destruction of acetyl choline, a process believed to account for the short-lived effect of a nerve

\* This paper also belongs to the series of investigations by the author and co-workers as "Studies in histochemistry XIV"

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stimulus (8) Furthermore nervous tissue, which liberates acetyl choline upon stimulation, is the richest source of choline esterase in the animal body In the present instance it has been found that by far the greatest concentration of the enzyme occurs in the epithelial cell layer of the gastric mucosa, and relatively little in the muscle This would tend to lend weight to the possibility that vagus stimulation causes the acetyl choline to be liberated chiefly in the epithelial cell portion of the mucosa Direct evidence, however, awaits future developments

It was the purpose of the present investigation to determine the quantitative distribution of choline esterase through the stomach wall of the pig in normal fasting and fed animals, and in those which had been given a previous injection of acetyl choline, eserine, or atropine A correlation was drawn between enzyme activity and the cellular composition of the tissue at any point

#### EXPERIMENTAL

##### *Procedure for the Study of the Enzyme Distribution in Stomach*

The method of sampling the tissue, obtaining serial microtome sections of the frozen material, and the extraction of the enzyme were analogous to the procedures used for study of other enzymes (1 and 4-6) The tissue from the freshly killed animal was stored at  $-12^{\circ}$  and the experiments were performed within 1 to 2 days Under these conditions no demonstrable loss in activity occurred Two consecutive circular sections, each  $25\mu$  thick, and having a diameter of 2.6 mm were placed in 9.2 c mm of 30 per cent glycerol in each reaction tube After extraction at room temperature for 2 hours, 16 c mm of M/10 veronal buffer containing  $\frac{1}{2}$  per cent acetyl choline chloride were added and the mixture was stirred by means of the magnetic "flea" in usual manner The reaction was allowed to proceed at  $30^{\circ}$  for  $1\frac{1}{2}$  hours, in the case of cardia and fundus, but for only 1 hour with pylorus tissue because of its greater activity Enzymatic hydrolysis was brought to a standstill, and the titration carried out with N/20 HCl to an end point at pH 6.2 according to the method already described (9)

Control experiments at  $30^{\circ}$  were conducted to determine the non-enzymatic splitting by placing the substrate-buffer mixture as a drop on the side of the reaction tube containing, but not in contact with, the glycerol solution with the tissue sections

##### *Extractability of the Enzyme*

In order to be certain that the 2 hour extraction of the sections was sufficient, an experiment was performed in which alternate deter-

minations were made upon tissue extracted for 2 and 4 hours respectively (Fig 1) From the manner in which the points lie on the curve it is apparent that complete extraction has occurred in 2 hours

It is of interest to obtain some idea of the tenacity with which choline esterase is bound to the tissue For this purpose an experiment was conducted in which estimations of activity were carried out alternately on sections treated with 30 per cent glycerol in the usual manner, and with 0.9 per cent NaCl solution for 2 hours Fig 2 shows the results of this experiment, and it may be seen that the physiological salt solution serves as well as the 30 per cent glycerol

It should be borne in mind that the greatest dimension of the various stomach cells lies approximately between 10 and 20 $\mu$ , and the 25 $\mu$  sections of frozen tissue must contain a considerable percentage of cells that have been cut open This may account in part for the ease of availability of the enzyme

The possibility should not be overlooked that in the experiment described above the enzyme may not have been extracted at all, but the substrate may have diffused into the cells and around the portions of the cut cells To demonstrate whether the enzyme is actually removed from the tissue, it would be necessary to separate the sections from the liquid medium, and then determine the activity of this liquid This has been done in the following fashion

Estimations of activity were conducted alternately upon sections treated with 30 per cent glycerol in the usual manner, and upon sections placed first in 0.9 per cent NaCl solution for 2 hours and subsequently in 30 per cent glycerol for the same length of time In the latter case the sections were removed from the saline solution with a glass needle and transferred to separate tubes containing 9.2 c mm of 30 per cent glycerol 6.5 c mm of the saline solution were used, and after removal of the tissue, 2.7 c mm of pure glycerol were added to make a final concentration of 30 per cent in a volume of 9.2 c mm The quantity of enzyme in this saline glycerol solution was determined, and another determination was made on the glycerol solution with the transferred sections The results of these experiments are represented in Fig 3 The magnitude of the activities observed in the saline solutions is a little less than the actual activities since a film of the saline extract remains on the surface of the

sections when they are transferred to the glycerol solution. Most of the enzyme appears in the saline solution, and the sum of the activities observed in this and the subsequent glycerol solution equals, or exceeds to some degree, the activities obtained by the single glycerol treatment alone. It would appear from this that choline esterase in the source in question is easily diffusible under the experimental conditions chosen, and is extracted from the tissue even by physiological salt solution.

In this connection it is interesting to note that Dale and Dudley (10) observed a rapid disappearance of acetyl choline from spleen when the tissue was minced, though little of the substance was lost if the organ was allowed to remain intact until ground for analysis of acetyl choline. Apparently the *mincing* process brought the esterase and acetyl choline into contact with one another, from which it might appear that either the enzyme and substrate are normally separated within the cell, or that the disruption of the cell resulted in activation of the enzyme or formation of the substrate from some precursor or both.

### *Histological Correlation*

In order to determine from which part of the mucosa the titrated serial sections were obtained, the tissue remaining after the sample had been removed for enzyme study was fixed, mounted, sectioned, and stained as described by Linderstrøm-Lang, Holter, and Sjøborg Ohlsen (2). The cellular composition of the stained sections was correlated with the titration curves (2), and the latter were labeled accordingly (Figs 1-13). The designations on the curves represent the positions where the respective cells occurred in greatest number. Cell counts were not performed in the present case since the epithelial cell region, which is obviously the one of greatest interest in relation to choline esterase, was rarely sufficiently intact in the stained sections to permit suitable counting.

### RESULTS

#### *Experiments on Stomachs from Normal Fasting Animals*

The tissue used in these experiments was obtained from pigs which had been starved for 24 hours previous to killing. Figs 2-4 demon-

strate the distribution of choline esterase in the cardia, fundus, and pylorus. In every case the highest enzyme concentration was found in the region where the epithelial cells predominate. In some instances a dip in the curve occurs beyond the epithelial portion followed by a rise in the chief cell region (Fig. 2). However, with other

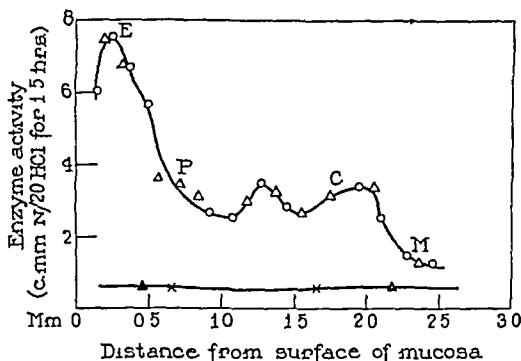


FIG. 1. On all the curves E refers to region of maximum concentration of epithelial cells. C refers to region of maximum concentration of chief cells, M refers to beginning of muscle layers, and P fundus refers to region of maximum concentration of parietal cells. Abscissae of all curves millimeters from surface of mucosa. Ordinates of all curves, hydrolysis (c.mm N/20 HCl after 1.5 hours in cardia and fundus, 1 hour in pylorus). Choline esterase distribution in fundus 88. Animal injected with 150 mg acetyl choline chloride 1 hour before killing.

- O = sections extracted for 2 hours
- X = controls
- Δ = sections extracted for 4 hours
- ▲ = controls

samples of tissue no dip was observed, but a steady decline followed to the region of the chief cells, and usually a further decrease in activity was demonstrable in the muscle tissue. The pylorus contained the enzyme in greater concentration than other parts of the stomach since practically as much activity was observed in 1 hour with this tissue as in 1.5 hours with the other tissues.

*Experiments on Stomachs from Normal Fed Animals*

The animals used for these experiments had been allowed to feed on their usual diet up until the time they were killed. The stomachs were found to be well filled with food. Figs 5 and 6 demonstrate typical curves for this material. The curves are essentially the same as those obtained using stomachs from fasting animals.

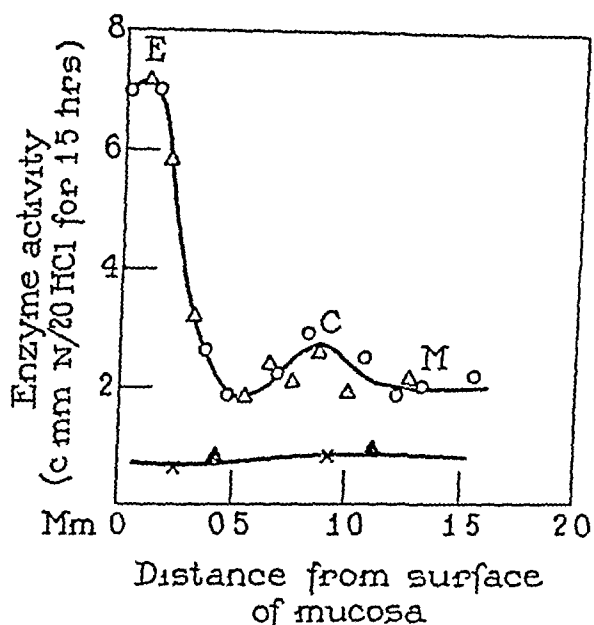


FIG 2 Choline esterase distribution in cardia 94 Normal fasting animal

○ = sections extracted with 30 per cent glycerol

× = controls

△ = sections extracted with 0.9 per cent saline

▲ = controls

*Experiments on Stomachs of Animals Injected with Acetyl Choline*

Acetyl choline chloride in freshly prepared aqueous solution was injected intramuscularly in doses of 100 or 150 mg into the hind-quarters of fasting animals 30 minutes and 1 hour respectively before the animals were killed. Reaction to the drug was apparent at the time the animals were slaughtered as indicated by excessive salivation and the presence of more than the usual amount of fluid in the otherwise empty stomach. The data obtained (Figs 1 and 7-9) do not differ actually either qualitatively or quantitatively from those of the normal un.injected animals. The broad maximum shown in

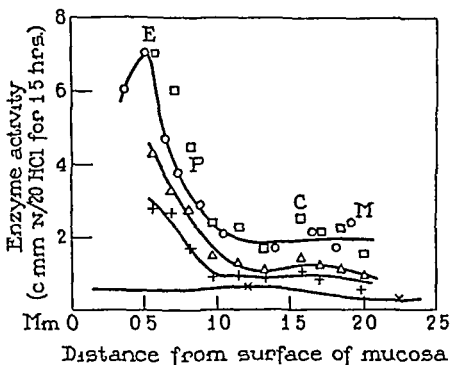


FIG 3 Choline esterase distribution in fundus 94 Normal fasting animal

- O = sections extracted with 30 per cent glycerol  
 X = controls  
 Δ = first extract with 0.9 per cent saline  
 + = subsequent extract with 30 per cent glycerol  
 □ = combined (Δ) and (+)

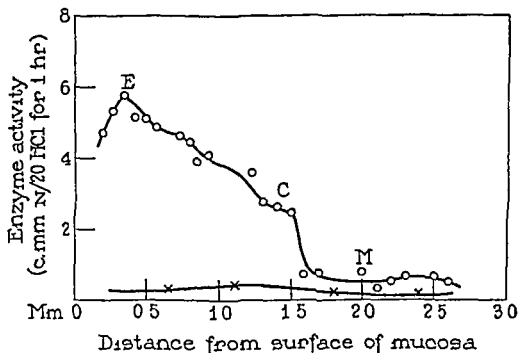


FIG 4 Choline esterase distribution in pylorus 82 Normal fasting animal

In Figs 4-13, O = total activity and X = control



Fig 9 is the effect of the presence of an abnormally great number of epithelial cells throughout the tissue to a depth of 1.5 mm

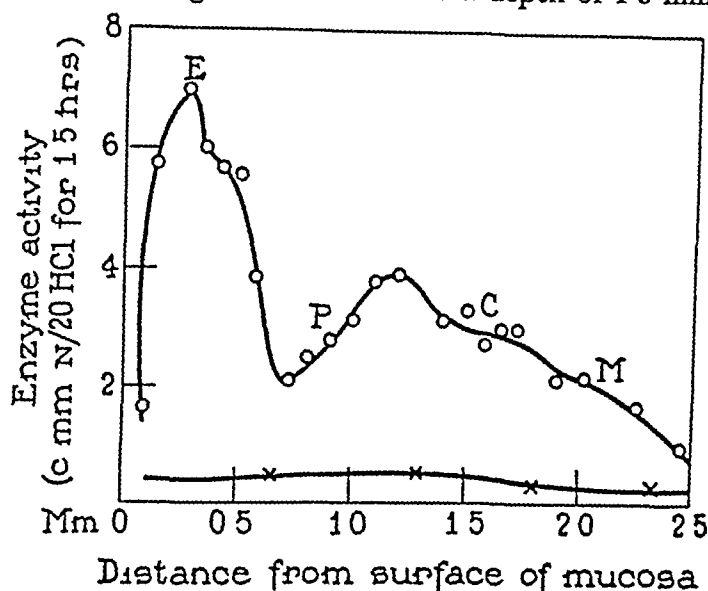


FIG 5 Choline esterase distribution in fundus 84 Normal fed animal

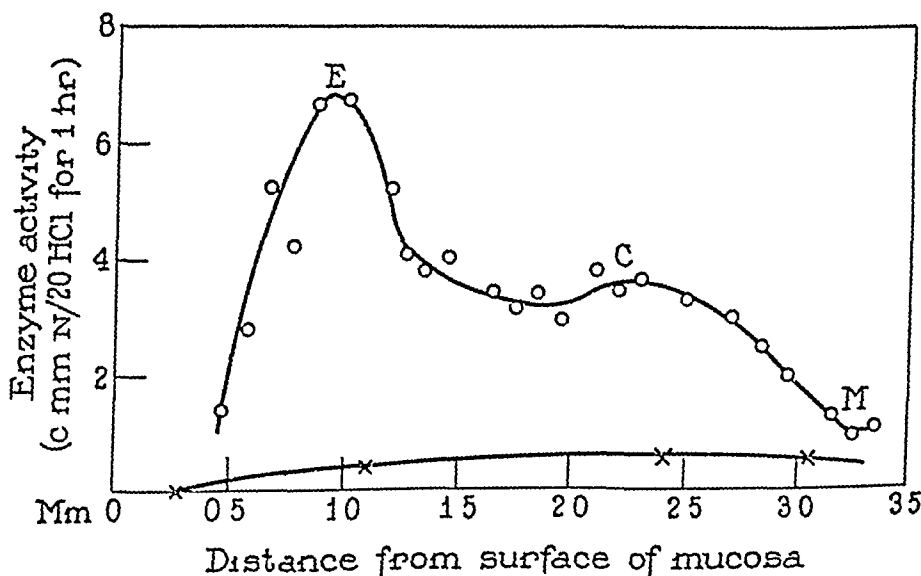


FIG 6 Choline esterase distribution in pylorus 84 Normal fed animal

### *Experiments on Stomachs of Animals Injected with Atropine*

As in the case of acetyl choline, atropine was injected intramuscularly into fasting animals 30 minutes before they were killed, the dose

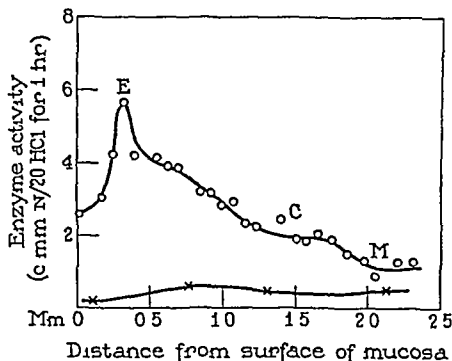


FIG 7 Choline esterase distribution in pylorus 88 Same as used for data in Fig 1

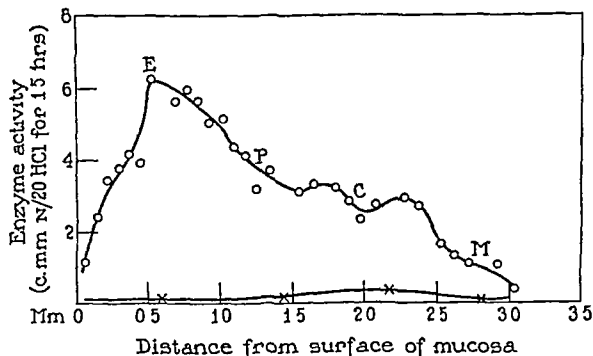


FIG 8 Choline esterase activity distribution in fundus 83 Animal injected with 100 mg acetyl choline chloride  $\frac{1}{2}$  hour before killing

used was an aqueous solution containing 200 mg of atropine sulfate. At the time of killing, the effect of the drug was evident by the dryness in the mouth of the animal and the presence of relatively little

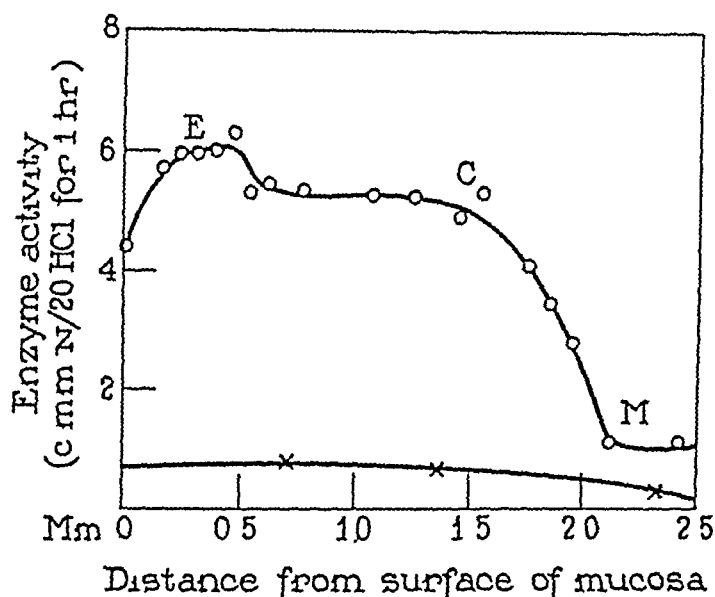


FIG 9 Choline esterase distribution in pylorus 83 Animal same as used for data in Fig 8

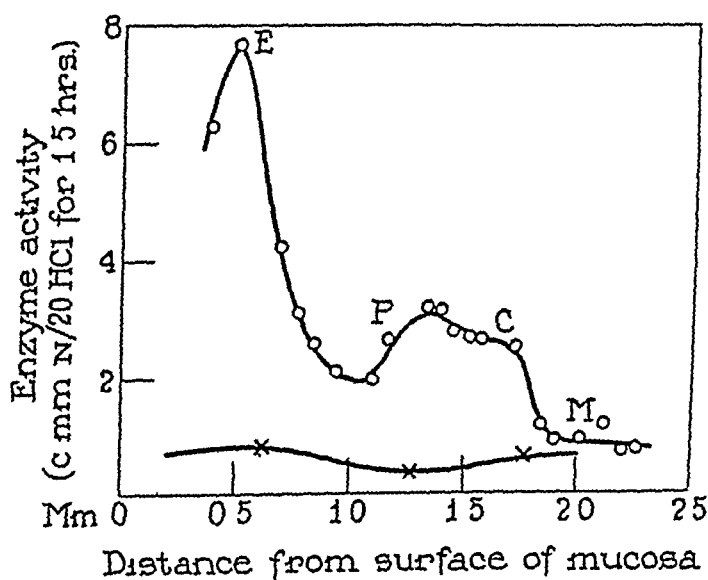


FIG 10 Choline esterase distribution in fundus 92 Animal injected with 200 mg atropine sulfate  $\frac{1}{2}$  hour before killing

fluid in the stomach, but the results presented in Figs 10 and 11 show no real deviation from the normal picture

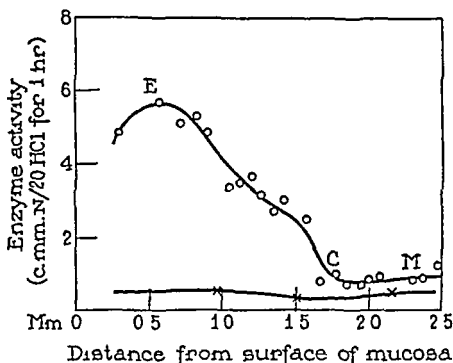


FIG 11 Choline esterase distribution in pylorus 92 Animal same as used for data in Fig 10

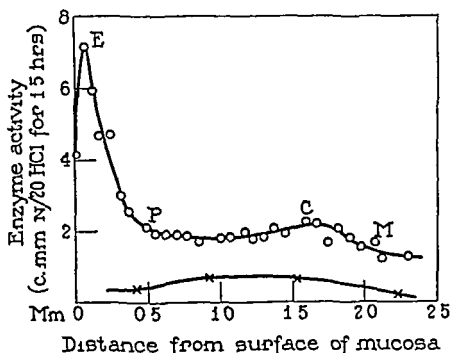


FIG 12 Choline esterase distribution in fundus 91 Animal injected with 30 mg eserine sulfate  $\frac{1}{2}$  hour before killing

#### *Experiments on Stomachs of Animals Injected with Eserine*

Fasting pigs were given the injection intramuscularly as usual, and after 30 minutes were killed. The dose employed was 30 mg

of eserine sulfate contained in aqueous solution. The reaction to the drug at the time of killing was similar to that produced by 150 mg of acetyl choline chloride. Typical curves are given in Figs 12 and 13. In this case as well, no marked change from the normal can be observed.

A simple calculation enables one to estimate the quantity of acetyl choline chloride split per cubic millimeter of tissue per second in the various cell regions. Thus the epithelial cell portion of the tissue yields activities of about 6 c mm N/20 HCl under the conditions

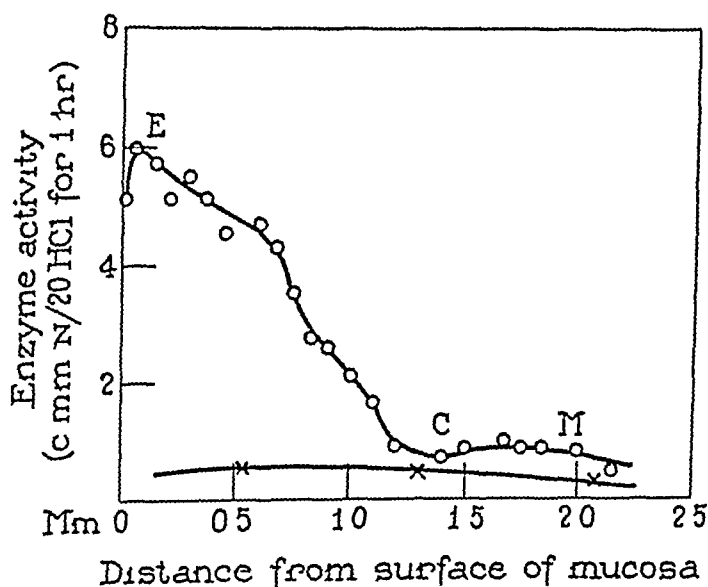


FIG 13 Choline esterase distribution in pylorus 91. Animal same as used for data in Fig 12.

given, we may take  $55\gamma$  as the corresponding approximate quantity of acetyl choline chloride split ( $1 \text{ c mm N/20 HCl} \approx 9.05\gamma$ ). The volume of two  $25\mu$  sections,  $2.6 \text{ mm}$  in diameter, is  $0.265 \text{ c mm}$ . Hence  $55\gamma \cdot \frac{1}{3600} \cdot \frac{1}{0.265} = 0.058\gamma$  split per second per cubic millimeter in pylorus, and  $\frac{2}{3}$  this or  $0.039\gamma$  in fundus and cardia.

#### DISCUSSION

The results presented all demonstrate the relatively intense choline esterase activity associated with the epithelial cell portion of the

mucosa. Curves of methyl butyrase activity in the pig stomach (5) also showed relatively greater activity in the region of the greatest concentration of epithelial cells, but the magnitude of the enzyme hydrolysis was equivalent to 2.0–2.5 c mm N/20 HCl in 5 hours at 40°, while for choline esterase the corresponding activity was found to be 6.0–7.0 c mm N/20 HCl in 1.5 hours (1 hour in pylorus) at 30°. The pH (8.7) was a little above the optimum in the former, and a little below (8.0) in the latter case for the respective enzymes. The greater activity of choline esterase as compared to methyl butyrase was characteristic of all cells in the stomach.

No consistent profile was found in that portion of the curves beyond the epithelial cell region. In some samples of tissue the activity fell off and maintained a steady low value to the muscle layer, while in others a small increase in the locality of the chief cells was observed. Although it is not certain without detailed cell counts on each specimen, it would appear from the stained sections that the presence of epithelial cells in greater or lesser numbers is the basis of these variations.

The higher concentration of epithelial cells in the pylorus doubtless contributes much to the consistently greater choline esterase activity in this portion of the stomach. Crypts lined with these cells extend much deeper into the stomach wall in the pylorus than in the fundus or cardia regions. It is not uncommon to find epithelial cells close to the muscle layer. The possibility that the high choline esterase activity associated with the epithelial cell material may be the result of the presence of this enzyme in the interstitial tissue rather than in the cells, or in both, should not be overlooked.

It might be expected that choline esterase would be mobilized in response to the appearance of acetyl choline in the tissue. However, this does not appear to be the case since the injection of acetyl choline, in quantities sufficient to produce a reaction in the stomach, resulted in no observable change in the enzyme activity. From this, and the fact that the normal stomach in either the resting or actively digesting state shows no essential difference in choline esterase concentration, the suggestion would seem pertinent that a fairly constant concentration of the enzyme exists permanently in the tissue in question, and that this concentration is independent of such factors

as nervous or direct chemical stimulation. Weight is lent to this suggestion by the findings that the known inhibiting effect of eserine upon the choline esterase in circulating blood (11-12) does not apply apparently to the gastric mucosa under the conditions herein described, no changes in enzyme activity of significance were elicited by the eserine administered, and furthermore atropine gave no demonstrable variation from the normal picture.

#### SUMMARY

Extraction experiments demonstrated that choline esterase could be removed from microtome sections of tissue with as great facility by 0.9 per cent NaCl as by 30 per cent glycerol.

The quantitative distribution of choline esterase through the wall of the pig stomach was studied, and it was found that the epithelial cell region possessed the greatest activity and muscle tissue the least. Pylorus was more active than fundus or cardia.

The enzyme activities found were independent of the physiological state of the normal stomach at the time the animal was killed.

Neither intramuscular injection of acetyl choline, eserine, nor atropine shortly before killing had significant influence upon the activity in any region of the stomach.

The implications of these results were discussed.

The author wishes to express his appreciation to Professor S. P. L. Sørensen for his interest in this investigation, to acknowledge his indebtedness to Dr. K. Linderstrøm-Lang for his invaluable suggestions and discussion, and to thank K. Mogensen for obtaining the stomachs and preparing and examining the stained tissue sections.

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# CRITICAL ILLUMINATION AND FLICKER FREQUENCY, AS A FUNCTION OF FLASH DURATION FOR THE SUNFISH

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## I

Interest of several kinds attaches to observations in which the relations of critical illumination ( $I$ ) and critical frequency of flashes ( $F$ ) for response to visual flicker are determined with varying proportions of light time to dark time ( $t_L/t_D$ ) in a flash cycle. Data of this kind should provide an important test of theories of visual flicker. The majority of reported experiments have employed a flicker cycle in which  $t_L = t_D$  (i.e., 50 per cent dark time). Measurements with  $t_L/t_D$  varied have given results which are to first inspection strangely contradictory. Porter (1902) and Ives and Kingsbury (1916) found for the human subject that with constant intensity in a flash while  $t_L/t_D$  was varied, the critical fusion frequency  $f$  passed through a maximum at or near  $t_L/t_D = 1$ . Hecht and Verrijp (1933-34) were able to give an equation requiring this effect on the basis of the general equation proposed (Hecht and Wolf, 1932-33) for Talbot's law, which accordingly became a test for the theory of the flicker curve obtained on the basis of that equation.

On the other hand it has been known for some time (cf. Piéron, 1922) that if observations are made by a method in which flashing is produced by the interruption of a beam of light, rather than by viewing a surface part white and part black upon which light falls, as in the experiments of Porter and of Ives and Kingsbury, the increase of  $t_L/t_D$  is accompanied by a progressive increase in  $f$ , the curve does not go through a maximum (Ives, 1922, Piéron, 1935). Moreover (Cobb, 1934), for "constant brightness at fusion"  $f$  de-

clines as  $t_L/t_D$  is increased, and for higher values of the ratio (and of  $I$ ) falls rapidly (*cf* also Piéron, 1928) These curves cannot be derived from the mechanism proposed on the basis of the theory that the data are primarily determined by the photochemistry of the retina (Hecht and Verrijp, 1933, 1933-34, Hecht, Schlaer, and Smith, 1935) The difference is presumably due to the effect of reflection from the "black" portion of the sector disc If this is correct, an adequate theory of the flicker function is required to account for (1) the increasing value of  $f$  as  $t_L/t_D$  is increased, with  $I$  constant, when flicker is produced by periodic cutting-off of light, and (2) the increase and decrease of  $f$  when  $t_D$  really represents time during which a lower intensity of light is acting, so that we have to do with cycles characterized by  $t_{I_1}/t_{I_2}$

From the standpoint of what may be called the intensity-discrimination theory of the marginal recognition of flicker (Crozier, 1935-36, Crozier, Wolf, and Zerrahn-Wolf, 1936-37*c, d*) the mechanisms in these two cases should differ significantly When  $I_1$  and  $I_2$  are measurable intensities, and by reflection from a sector disc  $I_2 = k I_1$ , the conditions for discrimination are not those which prevail when  $I_2 = 0$  The curve of  $I_c = \theta(f)$  is of the same form as that for  $I_c = \theta'(F)$ , as attested by experiments with the human eye in which  $t_L/t_D = 1$  (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b*)

Qualitatively it could be expected as reasonable, if the kinetics of light adaptation and dark adaptation, as directly measured, are immediately concerned in the determination of the flicker intensity contour, that raising the temperature and increasing the proportion of dark time in a flicker cycle should have the same direction of effect For a given energy of flash, the extent of dark adaptation should be increased at a fixed  $F$ , and  $I_c$  could then be smaller The detailed comparison of the  $F$ - $I$  contours obtained with temperature and  $t_L/t_D$  as parameters permits the evaluation of this notion It also tests the idea that Talbot's law really has anything to say about the properties of  $I$  and  $t$  for marginal flicker

Observations at various temperatures have been made with lower animals, by a technique which when used with human observers gives the well known properties of the flicker curve (Crozier, Wolf, and Zerrahn-Wolf, 1936-37*c, d*, 1937-38 *b*) The same procedure is

easily adapted for experiments in which the ratio  $t_L/t_D$  is varied. The present paper deals with our experiments on the sunfish *Enneacanthus gloriosus*.

## II

The apparatus is essentially a glass cylinder with black and transparent alternating stripes (Wolf and Zerrahn Wolf 1935-36 Crozier, Wolf and Zerrahn Wolf 1936-37a 1937-38a). The cylinder encloses a small aquarium containing the reacting animal, is evenly illuminated from outside, and is rotated at a known speed. The number and the widths of the opaque stripes can be such that any desired proportion of light time to dark time is obtainable in a cycle passing a given point at the cylinder wall. It is not practicable to have  $t_L/t_D$  less than 0.1 or greater than 0.9, owing to mechanical difficulties in the precise adjustment of very narrow stripes.

The responses of the fishes have been discussed in preceding papers (*cf* Crozier, Wolf, and Zerrahn Wolf, 1937-38a and earlier articles there cited). Ten individuals were used throughout the present series of observations. The method of treatment of the data has been described in the previous papers: three successive measurements of  $I$  with each individual are averaged to give  $I_1$ , the mean of these ten =  $I_m$ .  $PE_{I_1}$  is the P.E. of the dispersion of the ten. The  $t_L/t_D$  ratios used were 0.10, 0.25, 0.50, 0.75, and 0.90. At 0.50 a sufficient number of determinations were made to show that the sunfish of the present lot gave values of  $I_m$  very closely agreeing with those obtained from previous lots at the same temperature (21.5°). In Table I the entries under  $t_L/t_D$  not in bold face type are taken from earlier papers. The variation of  $I_1$  is discussed in Section IV. The observations at each value of  $t_L/t_D$  were taken in one series. The same ten individuals were used throughout. No progressive changes in sensitivity were detectable.

Careful examination has shown that in the new series of measurements, as in the older sets, the behavior of  $\sigma_{I_1}$  is of a uniform nature: the between-animal variation, except at the very highest flash frequencies, exceeds the within-animal; the relative sensitivities of the ten fishes fluctuate at random from one set of readings to another. The graph of  $\log PE_{I_1}$  vs  $\log I_m$  breaks at about the point where the declining rod function fades out; beyond this intensity the data can still be described as  $\sigma_{I_1}$  and  $I_m$  in direct proportion, but with a new origin (Crozier 1935-36).

The absolute values of  $PE_{I_1}$ , however, are lower than in our previous series at this temperature (21.5°). The reasons for this are unknown, but are probably found in the differences between the lots of sunfish. In Fig. 13 only the new data for  $t_L/t_D = 1$  are plotted (*cf* Table I).

The device producing flicker has one theoretical defect: the inner face of each opaque stripe receives light from the transparent bars on the opposite side of the cylinder. The reflection coefficient of the flat black paper is small, and the results demonstrate that under our conditions  $F$  does not go through a maximum as

TABLE I

$I_m$  (millilamberts) and  $P E_{I_1}$ , as  $\log_{10} I$ , at different flash frequencies  $F$ , for various percentages of light time ( $t_L$ ) in a flicker cycle. Observations on the sunfish *Enneacanthus gloriosus*, at 21° 5'. The  $\log I_m$  figures not in bold-faced type are from earlier experiments (Wolf and Zerrahn-Wolf, 1935-36, Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *a, d*), for these,  $N = 12$  individuals,  $n = 3$  observations on each at each  $F$ . For the newer data (bold-faced),  $N = 10$ ,  $n = 3$ .

$t_L$ percent	10		25		50		75		90	
$F/sec$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$
2							<b>7</b> 7013	<b>8</b> 4072	<b>7</b> 9718	<b>8</b> 4387
3			<b>7</b> 5702	<b>8</b> 4620	<b>7</b> 6555	<b>8</b> 8414	<b>6</b> 0795	<b>8</b> 7095	<b>6</b> 3418	<b>8</b> 6626
4			<b>7</b> 7827	<b>8</b> 3510	<b>6</b> 1784	<b>8</b> 716	<b>6</b> 5971	<b>8</b> 9070	<b>6</b> 8577	<b>7</b> 0827
5	<b>7</b> 8308	<b>8</b> 5348	<b>7</b> 9616	<b>8</b> 4294	<b>6</b> 3701	<b>7</b> 6010	<b>6</b> 8326	<b>7</b> 2014	<b>5</b> 0892	<b>7</b> 6229
6	<b>6</b> 9900	<b>7</b> 1655	<b>6</b> 7816	<b>7</b> 3249	<b>5</b> 2385	<b>6</b> 4814	<b>5</b> 6573	<b>7</b> 9987	<b>5</b> 9262	<b>6</b> 1052
	<b>6</b> 5630	<b>7</b> 0445			<b>6</b> 2423	<b>7</b> 6163				
7	<b>5</b> 5327	<b>6</b> 0983	<b>5</b> 7569	<b>6</b> 1436	<b>4</b> 1855	<b>5</b> 4456	<b>4</b> 6172	<b>6</b> 9682	<b>4</b> 8685	<b>5</b> 3430
8	<b>4</b> 3877	<b>6</b> 9813	<b>4</b> 5210	<b>6</b> 8914	<b>4</b> 9954	<b>4</b> 2788	<b>3</b> 4002	<b>5</b> 8909	<b>3</b> 6690	<b>4</b> 0378
					<b>4</b> 9848	<b>5</b> 3952				
9	<b>3</b> 1744	<b>5</b> 7345	<b>3</b> 3708	<b>5</b> 9442	<b>3</b> 7983	<b>3</b> 0934	<b>2</b> 1967	<b>4</b> 3681	<b>2</b> 4501	<b>4</b> 7668
10	<b>3</b> 9263	<b>4</b> 3495	<b>2</b> 0878	<b>4</b> 6497	<b>2</b> 6625	<b>4</b> 9225	<b>2</b> 9751	<b>3</b> 2100	<b>1</b> 2442	<b>3</b> 7251
	<b>3</b> 8736	<b>4</b> 2266			<b>2</b> 5600	<b>3</b> 8710				
					<b>2</b> 9446	<b>3</b> 4175				
15	<b>2</b> 2312	<b>4</b> 9235	<b>2</b> 5009	<b>3</b> 2060	<b>2</b> 9403	<b>3</b> 3581	<b>1</b> 3768	<b>3</b> 7309	<b>1</b> 6314	<b>2</b> 0934
					<b>2</b> 9543	<b>2</b> 1004				
20	<b>2</b> 4935	<b>3</b> 1380	<b>2</b> 8211	<b>3</b> 0342	<b>1</b> 2591	<b>2</b> 2480	<b>1</b> 6739	<b>2</b> 0390	<b>1</b> 9080	<b>2</b> 3130
25	<b>2</b> 7090	<b>3</b> 3662	<b>1</b> 1017	<b>3</b> 7359	<b>1</b> 5631	<b>2</b> 7993	<b>1</b> 9696	<b>2</b> 2721	<b>1</b> 2180	<b>2</b> 8904
					<b>1</b> 5566	<b>3</b> 8779				
30	<b>2</b> 9588	<b>3</b> 6142	<b>1</b> 3870	<b>3</b> 9046	<b>1</b> 8118	<b>2</b> 8639	<b>0</b> 2405	<b>2</b> 7498	<b>0</b> 5254	<b>2</b> 8024
35	<b>1</b> 2458	<b>3</b> 7615	<b>1</b> 7123	<b>2</b> 2949	<b>0</b> 1418	<b>1</b> 1106	<b>0</b> 5537	<b>2</b> 9262	<b>0</b> 8751	<b>1</b> 2978
	<b>1</b> 2405	<b>3</b> 6220								
40	<b>1</b> 6123	<b>2</b> 2438	<b>0</b> 0173	<b>2</b> 6572	<b>0</b> 4601	<b>1</b> 4487	<b>0</b> 8733	<b>1</b> 3724	<b>1</b> 3055	<b>1</b> 6356
	<b>1</b> .5738	<b>2</b> 0874								
45			<b>0</b> 3714	<b>2</b> 7937	<b>1</b> 0465	<b>1</b> 6180	<b>1</b> 4486	<b>0</b> 1038		
47							<b>2</b> 2660	<b>0</b> 3845		
50			<b>0</b> .9297	<b>1</b> 5120	<b>2</b> 2264	<b>1</b> 0618				
53			<b>2</b> 2927	<b>0</b> 4964						

$t_L/t_D$  is varied with  $I$  fixed. Hence the analysis of the flicker function can proceed without reference to this complication. This is true in our experiments with insects as well as for the sunfish.

## III

The observations are summarized in Table I. It is apparent that for a given  $I$  the intensity of a flash required for response decreases continuously as the flash duration is made less and the dark interval accordingly greater. At fixed intensity,  $F$  increases as  $t_L/t_D$  becomes smaller, and the maximum to which  $F$  rises is found to increase.

In Fig 1  $\log I_m$  is plotted as a function of  $F$ . The whole curve is shifted to lower intensities with decrease of  $t_L/t_D$ , and the maximum rises slightly. The curves show the features of structure already seen for  $t_L/t_D = 1$  (Crozier, Wolf, and Zerrahn Wolf, 1936-37 d, 1937-38 a). The form of the curve is not affected. Fig 2 demonstrates that the cone portion of each graph is accurately described by a probability integral. This is also true for the rod sections (Fig 3). The limitations of the apparatus are such that intensities greater than antilog 2.3 cannot be obtained, and on the other hand flash frequencies above  $F = 40$  could not be produced with adequate precision for  $t_L/t_D = 0.10$  or  $0.90$ . Below  $\log I = 7.5$  the animals cannot be clearly enough seen in the apparatus to observe their movements accurately. The curves are therefore incomplete in these respects. By trial, however, values of  $F_{max}$  are found for the cone parts which give an adequate description of the data (Fig 2). The further properties of the parameters of these descriptions give a sufficient justification for the procedure. A striking feature is that on the probability grid (with  $F$  as per cent  $F_{max}$ ) the slopes of the lines for the cone contribution are independent of  $t_L/t_D$ , and for the rod part also (Figs 2 and 3). From the standpoint that  $dF/d \log I$  vs  $\log I$  represents a frequency distribution, this means that  $\sigma'_{\log I}$  is independent of  $t_L/t_D$ , as it is of temperature (1936-37 d). In Fig 4 the graphs of Fig 2 have been moved on the  $\log I$  axis so as to coincide at  $F = 0.5 F_{max}$  (i.e., each value of  $I_m$  has been multiplied by a constant for each  $t_L/t_D$ ), the points form a band of statistically constant width on the  $\log I$  axis. The same procedure applied to the rod portions of the data is shown in Fig 5, here the relative departures of the points are greater on the  $F$  axis, although their absolute magnitudes are no greater (the maximum departure in Fig 5 is equivalent to  $0.63 F/sec$ ) since the  $F$  scale is magnified about seven times by comparison with that in Fig 4.

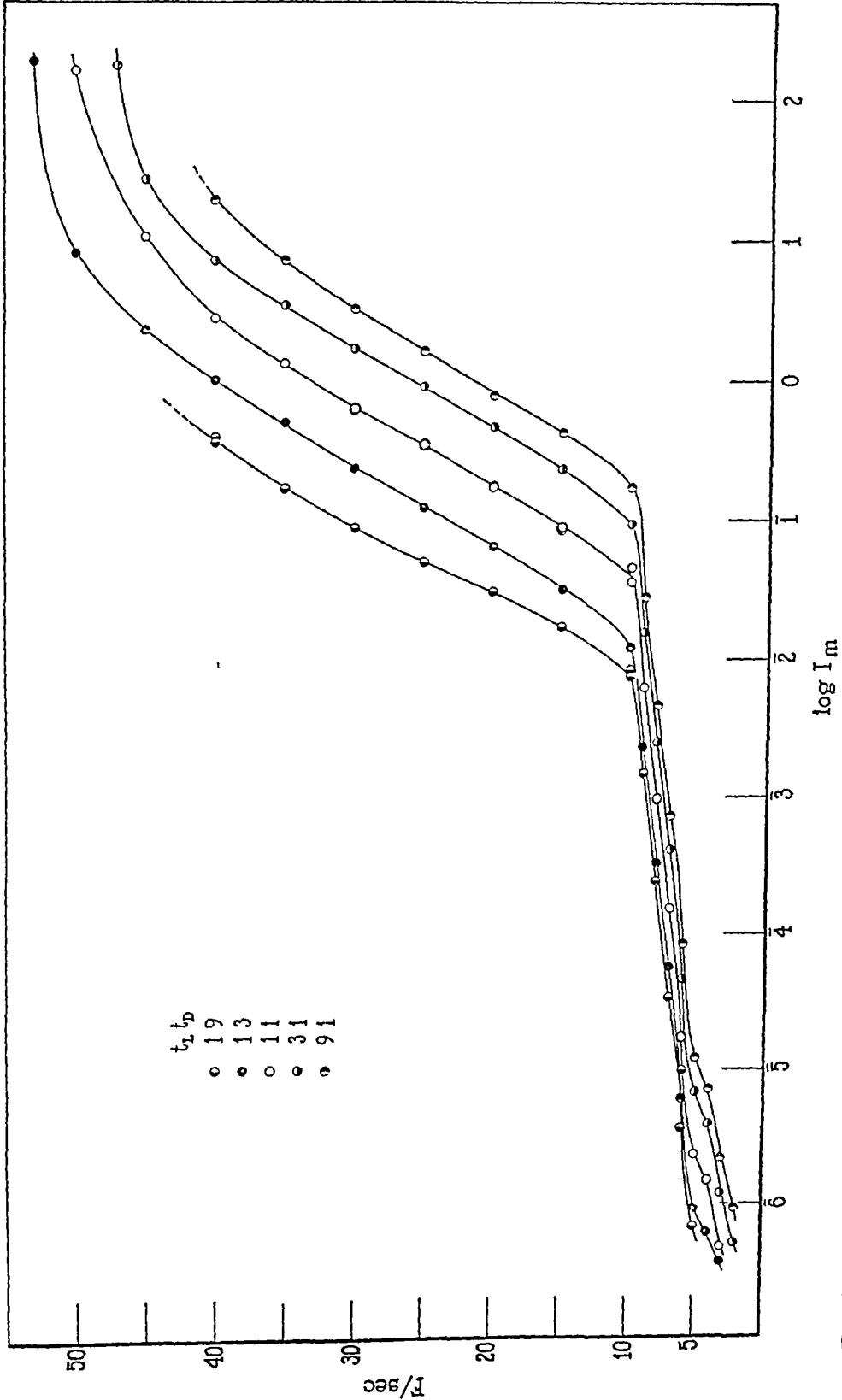


FIG 1 Flash frequency and  $\log I_m$  for various percentages of light time in a cycle, increasing from left to right Data on the sunfish *Enneacanthus* (Table I)

The description of the  $F - \log I$  curve by means of a probability integral (Crozier, Wolf, and Zerrahn Wolf, 1936-37 *d*, 1937-38 *a, c*, 1937, Crozier, 1937) has shown (1) that this function is adhered to by

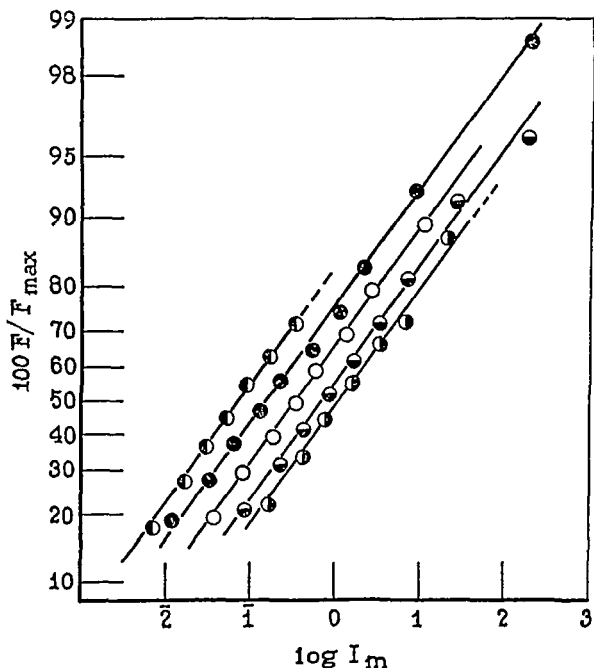


FIG 2 The upper (cone) portions of the curves in Fig 1 on a probability grid,  $F$  as  $100 F / F_{\max}$

the data, (2) that systematic deviations from it, in the case of certain insects studied, are readily accounted for by structural conditions (Crozier, Wolf, and Zerrahn Wolf, 1937-38 *b*), (3) that it provides a



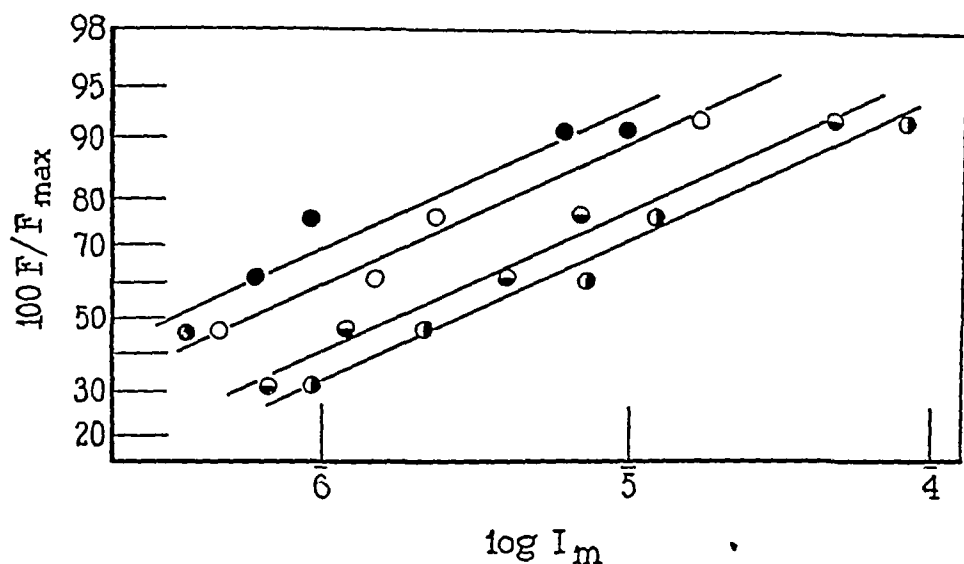


FIG 3 The lower (rod) ends of the curves in Fig 1 on a probability grid

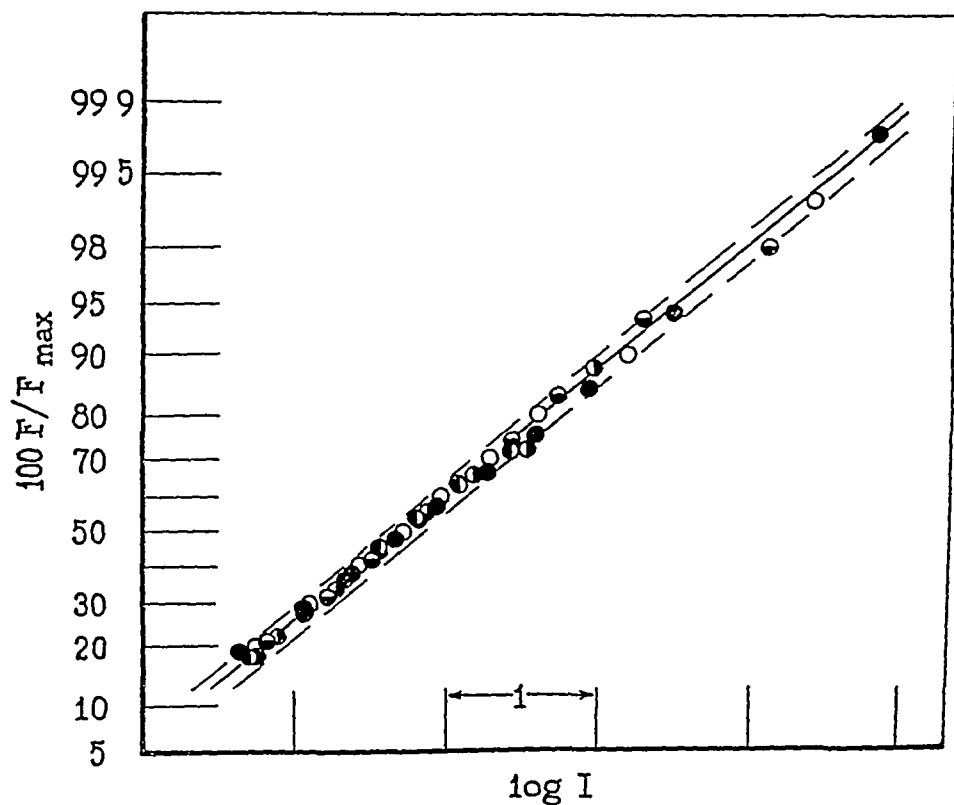


FIG 4 The cone curves of Fig 2 adjusted to the same value of  $\log I$  at the inflection point ( $F = 0.50 F_{max}$ ), the slopes are the same

rational dissection of the compound flicker curve for vertebrates, (4) that it is intimately connected with the intrinsic organic variability which is a basic feature of the data, (5) that its parameters bear the reasonably expectable relations to area and to temperature, which

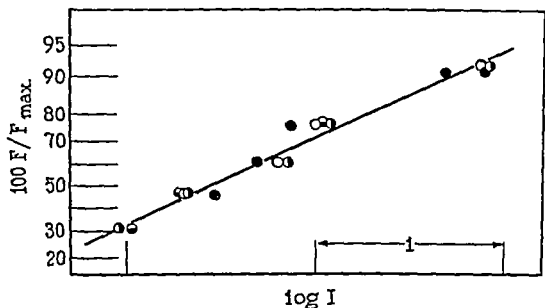


FIG 5 The rod curves of Fig 3 adjusted to the same value of  $\log I$  at the inflection point ( $F \approx 0.50 F_{\max}$ )

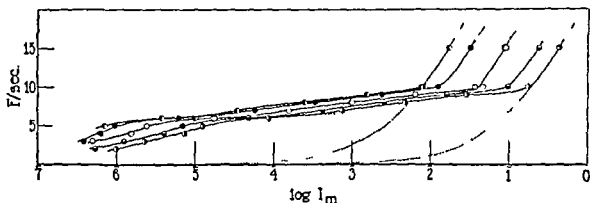


FIG 6 The lower part of the  $F - \log I_m$  plot (Fig 1), with the backward extensions of the probability integrals from Fig 2 (shown in two cases only) to demonstrate the manner in which the extrapolation to the neighborhood of  $F = 0$  accounts for the shape of the graph resulting from the summation of  $F$  elements due to rods and to cones

in the latter case would scarcely be looked for on other theoretical grounds, and (6), that the parameters in question are proved to have the nature of organic invariants determined by the composition of the organism (Crozier, Wolf, and Zerrahn Wolf, 1937)

We may consider first with the present measurements the composite character of the flicker curve. The rod contribution, with the various fishes we have studied, is of much lower maximum  $F$  than the cone part, the proportion  $M_R/M_C$  being much smaller (*ca* 0.13) than for man (*ca* 0.2). The  $\log I$  separation of the rod and cone branch (Fig. 1) is also greater, and the cone curve is steeper ( $\sigma_{\log I}$  less). This brings it about that with the fishes there is a flat region in the curve, almost horizontal, on which a small hump signifies the entrance of the effects due to cones, with the human data the overlapping is much more complete (Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *d*, 1937-38 *c*). Fig. 6 shows that, as for the curves at different temperatures (Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *d*), and for various genetic types (1937-38 *a*), the extrapolation of the cone probability integral back toward  $F = 0$  accounts precisely for the beginning of the upward drift of the observations at different levels of  $t_L/t_D$ .

The difference curves obtained by subtracting the extended cone integral from the observations are also rectilinear upon a probability grid (*cf* Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *d*, 1937-38 *a*).

#### IV

Upon the descriptive success of this formulation the contention rests that the theory of the marginal recognition of flicker must be derived from the properties of the parameters of the probability function. These properties are of two kinds. There are first the observable consequences of the fact that the curves (Figs. 2 and 3) describe the connection between  $F$  and *mean* values of  $I_c$ , the nature of the *variation* of  $I_1$ , which is lawful (Crozier, 1935-36, etc.), and which can be established through its quantitative relations to experimental variables, is also to be accounted for by any acceptable theory. Second, there are the properties which can be measured by expressing these parameters as functions of area, wave length, temperature,  $t_L/t_D$ ,  $t_{I_1}/t_{I_2}$ , and other conditions. The necessary parameters are three  $F_{max}$ ,  $\sigma_{\log I}$ , and  $\log I_m$  at the inflection point ( $= \log I_{inf}$ ). On a scale of  $F =$  per cent  $F_{max}$ ,  $\sigma_{\log I}$  is  $\sigma'_{\log I}$ . It is already found that  $F_{max}$  is decreased by decrease of visual area,  $\log I_{inf}$  increased,  $\sigma'_{\log I}$  apparently unchanged (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b*). With increase of temperature (Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *c, d*),

$F_{max}$  and  $\sigma'_{1\pm I}$  are unaffected,  $\log I_{i\mu}$  is decreased. By analogy with the case of electrical excitation (Crozier, 1937) we shall write  $\tau'$  for  $\log I_{i\mu}$

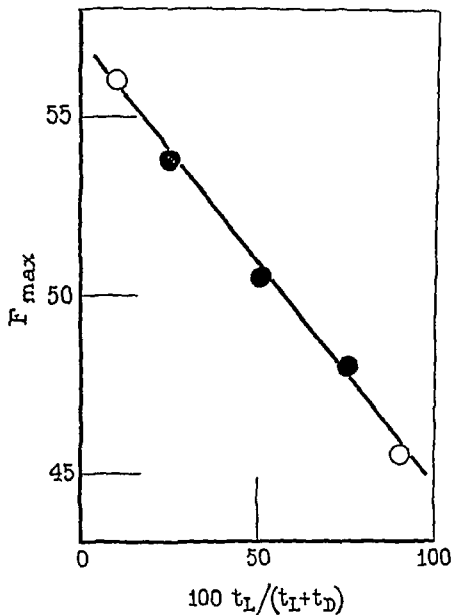


FIG 7  $F_{max}$  (cones) is a declining rectilinear function of the percentage of light time in the flash cycle. (The points at 10 per cent and at 90 are estimated from the calculations in Fig 2.)

With increase of  $t_L/t_D$  we find that  $F_{max}$  is lowered (Fig 1). The relationship is such that  $F_{max}$  is a declining rectilinear function of the percentage of the flicker cycle time occupied by light (Fig 7) (or, an increasing rectilinear function of  $t_D/(t_L + t_D)$ ). The effect is less

clearly evident with the rod curves, since a change in  $F_{max}$  of the same proportionate amount (23 per cent of  $F_{max}$  at  $t_L/(t_L + t_D) = 0.9$ ) would only be 1.6  $F$  units, and the detected change from 0.9 to 0.1 is less than this

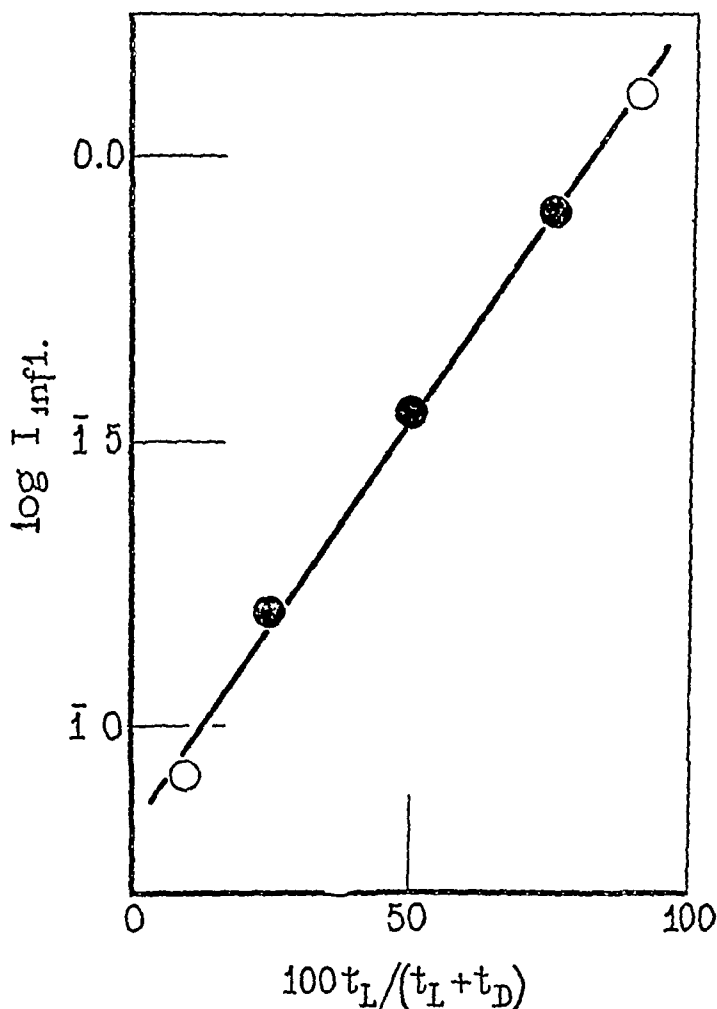


FIG. 8 The abscissa at the inflection point of the  $F$ ,  $\log I_m$  curves (cones) is a rectilinear function of the percentage light time

The value of  $\log I_{inf}$  ( $= \tau'$ ) is a rectilinear function of  $t_L/(t_L + t_D)$ , for both rod and cone curves (Figs 8 and 9). The proportionality constant is identical for the two sections, we may anticipate a subsequent report on similar experiments with *Anax* larvae to point

out that this relationship is discovered there also, but that the slope of the  $\tau'$  vs  $t_L/(t_L + t_D)$  plot differs from that in Figs 8 and 9

We have thus a situation in which  $\sigma'_{log I}$  is independent of  $t_L/t_D$ , area, and temperature, while  $F_{max}$  decreases directly with increase of  $t_L/(t_L + t_D)$  and  $\tau'$  increases in this proportion and decreases with rise of temperature, whereas  $F_{ms}$  is independent of temperature. To reconcile these facts with the conception that  $F$  and  $I$  are interdependent by reason of the nature of a (homogeneous) photostationary equilibrium of light adaptation and dark adaptation in the retina appears to be impossible. Certainly it cannot be done on the assumption that liminal flicker depends upon constant or constantly propor-

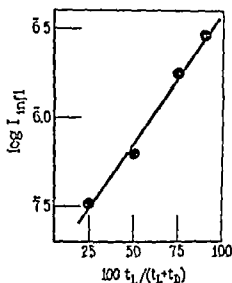


FIG 9 The rule observed in Fig 8 holds also for the rod portions of the flicker curves, and the slope constant is the same

tionate differences in the amounts or rates of photochemical change produced by alternating intervals of light and darkness

#### V

The  $F$ - $\log I$  curves have been compared for various ratios  $t_L/t_D$  on the basis of the intensity of the illumination during a flash. If Talbot's law were applicable to a situation of this kind, comparisons might be instituted on the basis of the equivalent mean intensity flux ( $I_{eq}$ ) at the point of reaction for each  $F$ . Instead of being brought closer together, however, the curves in Fig 1 are of course more widely spread apart when each  $I_m$  for  $t_L/t_D = 1/9$  is multiplied by 0.1, those for 1/1 by 0.5, and those for 9/1 are multiplied by 0.9,

while a transformation in terms of  $I \times t$  on the  $F$  axis inverts the order of the curves. Naturally, in discussing critical flicker frequencies, nothing can be said about equivalent brilliance in the case of lower animals, and nothing really justifies the use of Talbot's law at the critical flicker frequency.

Neither in terms of  $I_m$  nor of  $I_{eq}$  does  $F$  exhibit a simple relation to  $t_D$ , logarithmic or parabolic (as *cf* Piéron, 1935, etc.) As the

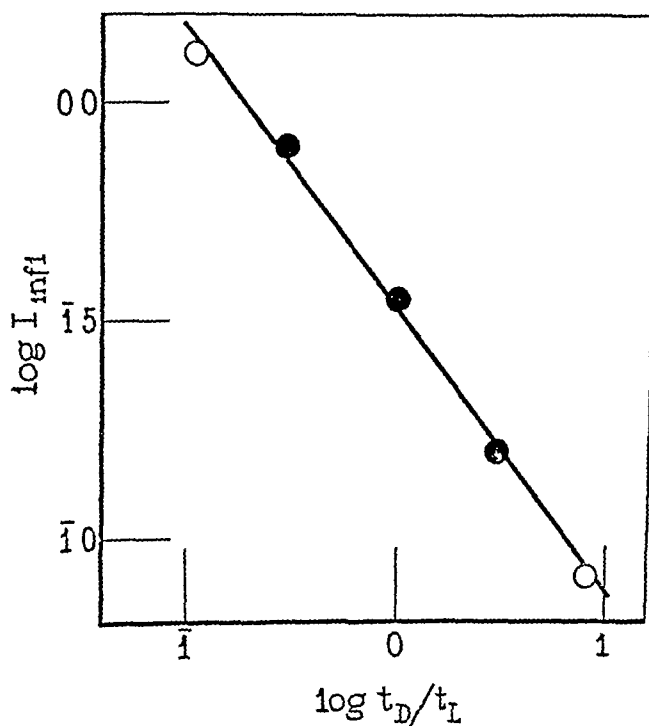


FIG. 10 For the cones,  $\log I_{nfl}$  is a rectilinear function of  $\log$  (dark ratio). This rule is not obeyed by the rod curves (Fig. 11), and is probably not a significant relationship. See text.

"dark ratio"  $t_D/t_L$  increases,  $F$  at fixed  $I$  also increases, but on a curve of which the shape depends on the magnitude of  $I_m$  or  $I_{eq}$ . For higher intensities,  $F$  is more nearly a straight line function of  $\log (t_D/t_L)$ . The nearest approach to a valid relationship of this kind is given by the fact that (over the range considered)  $\tau'$  is very nearly a straight-line function of  $\log (t_D/t_L)$ , for the cones, so that  $I_{nfl}$  is a decreasing parabolic function of the dark ratio (or, increasing, of the

light time ratio), as shown in Fig 10. The case is not so good for the rods (Fig 11). The same thing is found with  $Anax$ . Since (Figs 7 and 8)  $F_{max}$  is rectilinearly related to  $\tau'$ , the same relation must hold for it, with reversed sign (Fig 12), and also for  $F_{inf}$ , with  $F_{max}$ . the slope is not far from 1, so that  $F_{max} \cong 51 + \log_{10}(t_D/t_L)$ . In general the rule that  $F \propto \log(t_D/t_L)$  can be expected to hold only at the highest intensities, as a limiting condition.

These interrelationships at least indicate that the curves in Fig 1 reflect merely a change in the scale of the  $F$ - $\log I_m$  function when

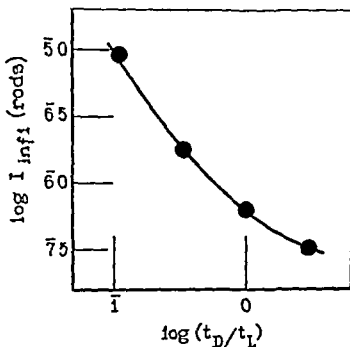


FIG 11 For the rod curves  $\log I_{inf}$  is not (as for the cones Fig 10) a straight line function of  $\log t_D/t_L$ .

$t_L/t_D$  is altered. They are consistent with the idea that one is really dealing with a population of elements, the additive effects of which are proportional to  $F$ , and that the sum of effects producing the marginal response to flicker depends in a particular way upon the proportion of light time in a flicker cycle. When  $t_L/t_D$  is low, the total population of elements is larger, its sum being  $F_m$ , and as it grows, in direct proportion  $\tau'$  is augmented,  $F_{max}$  and  $\tau'$  each being in rectilinear relation to the increase of the fraction of the total cycle occupied by light.

The reciprocal changes in  $F_{max}$  and  $\tau'$  are consistent with expecta-



tion When area is decreased,  $\tau'$  is higher and  $F_{max}$  less This effect is to be looked for when the total available number of elements has been reduced The legitimacy of considering  $F$  and visual area as equivalent in this way, that is, in terms of their significance for the magnitude of the sensory effect produced, is attested particularly by experiments below the critical flicker frequency for the bee two flickering fields have the same phototropic effect if the product of flicker frequency  $\times$  area is the same, evidence of the same kind is provided by *Limulus* (Wolf and Zerrahn-Wolf, 1934-35, 1936-37) When  $t_L/t_D$  is increased it is reasonably supposed that the total number of

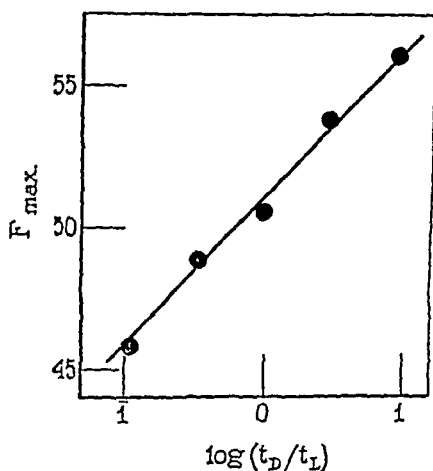


FIG 12 The maximum  $F$  to which the flicker curve rises is directly proportional to  $\log(t_D/t_L)$

available elements is made less, because recovery of any one end-organ (rod or cone) from the effect of a flash will be less probable if the flash is longer and the dark period less, at given cycle time ( $1/F$ ) Hence to achieve a given effect ( $F$ ),  $I$  must be increased  $\tau'$  is the measure of  $\log I_m$  required for activation of 50 per cent of all the available elements It could therefore be expected to bear a simple relation to the fraction of the cycle occupied by light, and it is found to be directly proportional to this fraction (Figs 8 and 9) If the sensory structures to be excited are the same, however,  $\sigma'_{\log I}$  should not be a function of the percentage of light time, any more than it is of temperature  $F_{max}$  is independent of temperature because the

total number of available elements is not affected, although the mechanism whereby the action of flashes is balanced against their after effects is influenced

The latter mechanism has been taken to be essentially that operating in the discrimination of intensities. In the flicker experiment only one intensity is involved, the adjusted intensity  $\tilde{I}_2$ , it is to be just discriminated from darkness,  $I_1 = 0$ , so that  $\Delta I = \tilde{I}_2 - I_1 = I_m$ . An essential attribute of  $\Delta I$  as obtained when  $\Delta I_m = \tilde{I}_2 - I_1$  is that  $\Delta I_m$  and  $\sigma_{1,1}$  are directly proportional (Crozier, 1935-36, 1936, Crozier and Holway, 1937, Holway and Crozier, 1937), and the ratio is not a function of area (*i.e.*, of number of sensory elements). In the data of critical flicker it has been shown that  $I_m$  and  $\sigma_{1,1}$  are in direct ratio (Crozier, 1935-36, Crozier, Wolf, and Zerrahn Wolf, 1936-37 *b, c, d*, 1937-38 *a, c*), and that the proportionality is independent of area (Crozier, Wolf, and Zerrahn Wolf, 1937-38 *b*). We here find also that it is independent of  $t_L/t_D$  (Fig. 13), and in *Anax* as well. The homologue for changes in  $t_L/t_D$  is found in certain experiments involving sensory discrimination of tension, the relation between  $\Delta W$  and  $\sigma_{1,\Delta W}$  is independent of frequency of the submission to tension, although increasing frequency decreases  $\Delta W$  (*cf.* Crozier and Holway, 1937).

The nature and the mode of action of the sensory elements concerned in this formulation can be investigated without assumptions as to their identity with single rods and cones. Elements are simply defined by  $dF/d \log I$ , at given  $I_m$  their sum is  $F$ . Identification with the physicochemical properties of individual rods and cones is unnecessary, and indeed impossible. The mode of action in the sensory discrimination of flicker has been pictured (Crozier, Wolf, and Zerrahn Wolf, 1936-37 *d*) as involving discrimination between (a) the central effects of the flashes of light and (b) the after images of these effects. It can be supposed that the rate of decay of the after-effect of a flash depends upon the rate and the duration of the building up of the photic effect, and upon an intrinsic velocity constant. If the discrimination of light (flashes) from non light intervals in cyclic succession depends upon the recognition of (reaction to) an efficient difference between (a) and (b), the relation of the  $F$ - $\log I$  curve to temperature is easily explained and a reasonable account is obtained

of the behavior of the variability of  $I_1$ , as we have shown. These properties are exhibited by the responses of animals possessing very different eyes structurally, and, as in the case of other phenomena

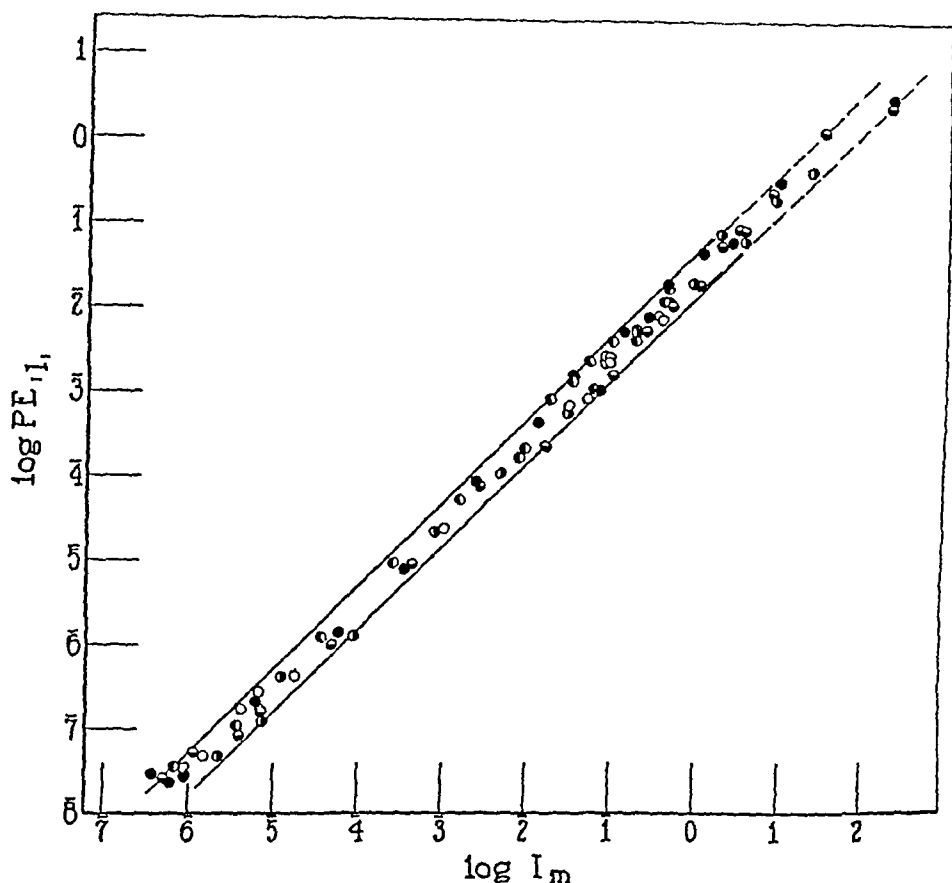


FIG. 13  $I_m$  and  $P E_{I_1}$  are directly proportional up to about  $\log I_m = 1$ , above that, as in other data on vertebrates, the slope on the log grid is less than 1. The proportionality constant is quite independent of  $t_L/t_D$ . The symbols are as in Figs 1, 2, etc.

of intensity discrimination, their essential dynamical features cannot be understood in terms of specificities of superficial structure.

To account for the changes in the  $F$ - $\log I_m$  curve as a function of  $t_L/t_D$  in these terms is not especially difficult. In the diagram of Fig. 14 there has been indicated schematically the time course of development of the mean effect of a flash ( $A-B$ ) which for  $t_L/t_D =$

1 is critical for response to flicker The effect  $E$  is due to the combined action of all the impulses peripherally produced during the action of

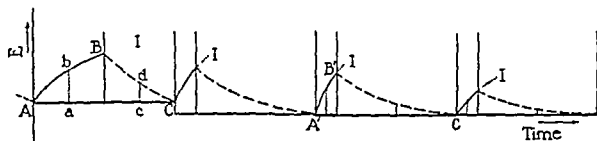


FIG 14 Schematic theoretical representation of events in a flicker cycle to show the way in which changes in the proportion of light time in a cycle can produce the observed changes in the  $F - \log I$  curve The time curve for development of sensory effect of a flash has a velocity constant which is a function of  $I$  and of the number of excitation elements available the decay curve for the after-effect at fixed temperature, has a fixed velocity constant

$AB$  is the course of development of the sensory effect  $E$  of a flash, at the critical point for response to flicker  $BC$  the decline of its after effect in an equally long dark interval. Between the mid-ordinates  $ab$  and  $cd$  (or the mean rates of change of  $E$ ) there obtains a certain difference which is necessary for response to flicker at this level of  $E$

If the cycle time ( $= 1/F$ ) be kept constant and the intensity in a flash and  $t_L$  is reduced say to 15 per cent, a new position of balance  $A'B'C'$  is arrived at, at a lower mean  $E$  although the velocity of increase of effect due to a flash is higher ( $I'$ ) with the same  $I$  because the number of elements of excitation is then larger (see text), but the difference between the mid point ordinates is now greater, and flicker is recognized (by response) Only by lowering the intensity ( $I''$ ), producing a less rapid rise of the effect of a flash, can a balanced rise and fall of effect with constant mean  $E$  be achieved which will permit a just adequate difference between the mid point  $E$  ordinates for the two parts of the cycle

(Similarly, for constant  $I$  in a flash the cycle time must be reduced for the same kind of adjustment)

Thus, with respect to the recognition of flicker when light and darkness alternate duration and intensity in a flash do not have reciprocal significance From the data of Table I plots can be made of  $\log I_m$  vs  $100 t_L/(t_L + t_D)$  at each flicker frequency, and by interpolation we can get the values of critical  $I$  for different durations of a flash ( $t_L$ ) at constant dark time The curve goes through a maximum for long  $t_D$ , and its shape is a function of  $t_D$  Talbot's law has nothing to say about the form of the critical flicker contour

the flash Its form must be primarily the result of the time and intensity-distribution of the excitabilities of the peripheral units and of their central representations  $B-C$  is the course of the decay

of the after-effect (*cf* Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *d*) For marginal response to flicker it matters little at the moment whether we take a critical difference between the areas under  $A B$  and  $B C$  or between the mid-ordinates as significant When  $t_L/t_D$  is made  $< 1$ , the number of available units is larger, as indicated by the analysis already given, therefore  $A B$  rises faster, consequently, with  $I$  held constant, a smaller cycle time is required to give the same kind of difference between the mid-point effects of the light and the dark intervals Similarly, for increase of  $t_L/t_D$  the number of potentially available elements is decreased, hence (at fixed  $F$ )  $I$  must be increased to give the same kind of balance In the first case, however, with decreased light time, the mean level of  $E$  is then less than when the light ratio is higher (Fig 14), this corresponds to the fact that the actual response, though "threshold," is weaker and less pronounced than when  $t_L/t_D$  is high The elements concerned in the exhibition of the response are elements having to do with intensive discrimination, not directly with the magnitude of sensory effect ("brightness") at which the discrimination takes place, no difficulty therefore arises in the addition of elements due to excitation of rods and of cones, since the locus involved is presumably central, not retinal

Certain features of the variability of  $I_1$  can on this theory be accounted for in the same general way as used for the data at different temperatures (Crozier, Wolf, and Zerrahn-Wolf, 1936-37 *d*) At a given  $F$  a higher  $I$  is required for reaction the longer the proportion of light time in a flash Therefore the greater the chance of moment-to-moment fluctuation in the mean effect produced by the flash From the relationship

$$\sigma_F = K \sigma_I (dF/dI)$$

(*cf* Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *a*) it can be seen that, for a given ratio  $t_L/t_D$ ,  $\sigma_F = K' dF/d \log I$ , the proportionality constant  $K'$  must be independent of  $t_L/t_D$ , consequently  $I_m$  and  $\sigma_{I_1}$  must be in the same proportion at all values of  $t_L/t_D$  (Fig 13)

To what extent this should be true when not light and darkness but two intensities of illumination are flashed in succession awaits investigation Measurements are needed in which  $I_1$  is not zero, but  $I_2$  and  $I_1$  are both finite intensities which are varied

## VI

## SUMMARY

From the relations between critical illumination in a flash ( $I_m$ ) and the flash frequency ( $F$ ) for response of the sunfish to visual flicker when the proportion of light time to dark time ( $t_L/t_D$ ) in a flicker cycle is varied at one temperature (21.5°) the following results are obtained

At values of  $t_L/t_D$  between 1/9 and 9/1 the  $F - \log I_m$  curves are progressively shifted toward higher intensities and lower  $F_{max}$ .  $F_m$  is a declining rectilinear function of the percentage of the flash cycle time occupied by light

The rod and the cone portions of the flicker curve are not shifted to the same extent

The cone portion and the rod region of the curve are each well described by a probability integral. In terms of  $F$  as  $100 F/F_m$ , the standard deviation of the underlying frequency distribution of elemental contributions, summed to produce the effect proportional to  $F$ , is independent of  $t_L/t_D$

The magnitude of  $\log I_m$  at the inflection point ( $\tau'$ ), however, increases rectilinearly with the percentage light time in the cycle

The proportionality between  $I_m$  and  $\sigma_{\tau'}$  is independent of  $t_L/t_D$

These effects are interpreted as consequences of the fact that the number of elements of excitation available for discrimination of flicker is increased by increasing the dark interval in a flash cycle. Decreasing the dark interval has therefore the same kind of effect as reducing the visual area, and not that produced by decreasing the temperature

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# CONCENTRATION AND PURIFICATION OF BACTERIOPHAGE

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## INTRODUCTION

Many of the reactions now known to be caused by enzymes were long considered to be "vital" processes and to differ qualitatively from ordinary chemical reactions. This point of view was held by many workers until Buchner succeeded in causing fermentation of sugar without living cells. This experiment (1) ended the controversy over the nature of "enzyme" reactions but at about the same time Iwanowski (2) and Beijerinck (3) discovered an entirely new class of "active agents" which have become known as filterable viruses and a new controversy, similar to the old enzyme controversy immediately arose. The bacteriophage first described by Twort (4) and by d'Herelle (5) represents one of this group of "active agents" and in many respects is more amenable to quantitative work than others. It possesses, in common with the other "viruses," the property of increasing in the presence of living cells and for this reason has been considered to be itself a living organism. It has been found that pepsin and trypsin under certain conditions also possess the property of increasing autocatalytically and there are, of course, many other known autocatalytic reactions. The criterion of increasing in quantity, or "growth" therefore does not distinguish sharply between enzymes and living organisms. This fact has been clearly recognized by Bordet (6) and his collaborators who considered the bacteriophage to be an abnormal product of metabolism. A similar point of view is held by Zinsser (7). Stanley (8) has recently shown that the virus of tobacco mosaic is, in all probability, a protein and it will be seen below that the bacteriophage is probably also a protein and that its



properties may be explained by much simpler assumptions than those involved in the view that it is a living organism

The most successful of the many attempts to purify and isolate bacteriophage was carried out by Bechhold and Schlesinger (9). Schlesinger (10) obtained from *B. coli* lysates a suspension of particles of about 90 m $\mu$  diameter which he considered to be the active agent itself. This preparation was extremely active and was free from bacterial protein but no convincing evidence of its purity was obtained. Bechhold (11) considered the particles to be living cells.

Most of the work has been done with small quantities of crude material and has been carried out entirely in solutions. Experience in the purification and isolation of enzymes has shown that it is essential to have in hand large enough quantities so that weighable amounts of solid material are available. Since even concentrated bacteriophage cultures contain only a few milligrams of organic matter per milliliter and since it is to be expected that the active principle is at most a few per cent of this amount it is evident that large quantities of culture must be handled. It is also essential to have an accurate method for activity measurements as otherwise small variations in activity cannot be detected and hence the progress of purification cannot be accurately followed. Large quantities of culture cannot be handled efficiently during chemical manipulation without contamination with other organisms so that it is also necessary to have a titration method which can be used in the presence of contaminating organisms. Pathogenic organisms present a further difficulty owing to the care with which they must be handled.

### *Cultural Methods Used*

The above considerations led to the selection of a culture of staphylococcus and its accompanying phage isolated by Glaser and by Shope (12) from house flies. The organism is non-pathogenic and the phage may be titrated by a modification of Krueger's (13) accurate titration method. Preliminary experiments showed that broth cultures contained so much inert material that purification was extremely difficult. Since the phage concentration is proportional to the bacteria concentration it should be possible to obtain very concentrated phage solutions by causing lysis to occur on agar cultures. This was found to

be true and washings from such lysed cultures may be many hundred times as active as broth cultures. They also were found to contain too much foreign material to handle.

It became evident that a synthetic media would have to be found but since staphylococci do not grow on the usual synthetic media a good deal of work was done before such a medium was obtained. It was eventually found that an extract of dried yeast in boiling water gave a practically protein free solution which was an excellent medium for growth. Conditions were worked out for the production of this media in 200 liter lots and for the growth and subsequent lysis of the culture in this quantity without previous autoclaving.

The lysed solution obtained in this way was slightly yellowish and clear. It contained about 10 [ph u] ml<sup>-1</sup>, about 0.02 mg protein nitrogen per ml, and some mucin like material. It could not be precipitated with neutral salts nor fractionated by any of the usual methods. This is usually the case with such dilute solutions, especially if they contain mucin. Some of the mucin like material was removed with lead acetate and the filtrate concentrated *in vacuo* at 30°C to 1/20 its volume. There was no loss in activity during this operation and the solution was now sufficiently concentrated so that the usual methods of protein fractionation could be used with some hope of success. A good deal of the mucin and inert protein could be removed by filtration with Filter Cel but the resulting solution still could not be fractionated. In the meantime it had been found that the active principle was not attacked by trypsin. Trypsin was therefore added to this filtrate and the solution allowed to stand at 10°C for several days. At the end of this time all of the inert protein had been digested and the solution could now be readily precipitated with 0.6 saturated ammonium sulfate. The precipitate obtained in this way is brownish. The color is removed by charcoal in alkaline solution and the protein reprecipitated with 0.6 saturated ammonium sulfate. The precipitate is filtered with Filter Cel to remove traces of charcoal and some inert protein. The details of the procedure are shown in

<sup>1</sup> One phage unit (ph u) is defined as the quantity of active agent which will cause lysis of 5 ml of standard suspension in 1 hour at 35°C. The usual broth lysates contain about one [ph u] ml on this scale. This unit is equal to about 10<sup>10</sup> ph u as defined by Krueger (13).

TABLE I

*Preparation of Bacteriophage*

No	Vol liters	[ph u]		[ph u] $\frac{\text{mg}}{\text{protein N}}$	[Protein N]	
		per ml	Total		per ml	Total
					mg	mg
1	200	1 0	200,000	50	0 02	4000
2	200	1 0	200,000	100	0 01	2000
3	10	20 0	200,000	100	0 20	2000
4	10	20 0	200,000	2000	0 01	100
5	10	20 0	200,000	5000	0 004	40
6	10	10 0	100,000	2500	0 004	40
7	10	10 0	100,000	5000	0 002	20
8	0 100	500 0	50,000	3000	0 16	16
9	0 100	500 0	50,000	5000	0 10	10

Suspend 2.5 kilo dry brewer's yeast in cheese cloth bag in 200 liters boiling water\* for 2½ hrs, remove yeast, adjust pH to 7.6, boil solution 3 hrs, cool to 37°C, and inoculate with staphylococcus suspension from 5 Blake bottles, add 5 × 10<sup>-4</sup> ml bacteriophage solution, bubble air through for 18 hrs, clear solution. Cool to 15°C, titrate to pH 9.0, add 1 liter M/10 lead subacetate and 300 ml M/3 pH 7.6 phosphate buffer and 100 ml chloroform. Allow to settle 24 hrs 15°C, supernatant. Supernatant evaporated at 30°C *in vacuo* to 7-10 liters, adjust pH to 7.6, cloudy. Add 20 gm Filter-Cel per liter and filter through 1450 ½ folded paper, filtrate. Add 500 mg crystalline trypsin, stand 10°C until 5 cc of solution gives flocculant precipitant when mixed with 10 ml saturated ammonium sulfate and allowed to stand 3-4 hrs at 10°C. This usually requires 48 hrs but may take several days. 5 + 2 vols (20 liters) saturated ammonium sulfate, stand 10°C 2-3 days, slight precipitate with clear supernatant. Siphon off supernatant. Precipitate suspension—add 5 vols N/10 ammonium hydroxide, dark brown solution. Add 5 gm Darco per liter. Filter 1450 ½, clear, colorless solution. Add 300 gm ammonium sulfate/liter and keep 2-3 days 10°C greyish white precipitate settles. Decant supernatant. Centrifuge precipitate suspension at 10°C. Dissolve precipitate with 10 times its weight of N/20 ammonium hydroxide. 8 + 2 gm Filter-Cel/100 ml. Filter No 3, suction.

\* Extracts of some preparations of dried yeast contain considerable amounts of protein. In the case of such yeast preparations it is necessary to add sufficient acetic acid (before adding the yeast) so that the resultant extract is about pH 4.5-4.7. Under these conditions all preparations of dried yeast used in this work yielded an extract containing less than 0.01 mg protein nitrogen per cc.

Table I which represents about an average experiment. The yield varies from 10-80 per cent of the original phage activity. It will be noticed that through the first five steps there is a rapid decrease in protein but no loss in activity and at this point the specific activity of the preparation ( $\mu\text{ph u/mg protein nitrogen}$ ) is the same as the final preparation. In succeeding steps, however, there is, at each precipitation, a loss in activity without loss of protein, *i.e.*, inert protein is formed. This loss of activity during the process of purification has greatly increased the difficulty of the work. Crude phage solutions are extremely stable and may be kept for years at  $0^{\circ}\text{C}$  without loss of activity. As purification proceeds the preparation becomes increasingly unstable, so much so that many of the usual methods cannot be used. Thus, filtration of any active precipitate results in loss of much activity and if the filtration be carried on until the precipitate is dry the activity is completely destroyed. Prolonged centrifuging inactivates the material and concentration in Wyckoff's ultracentrifuge also causes more or less complete inactivation. On the other hand, settling by gravity causes very little loss and this method is therefore used as much as possible.

The original concentration of active phage present in the crude culture may be calculated from the specific activity of the final product and is found to be about 1 mg per liter. This is very much less than that found by Schlesinger with *col<sub>1</sub>* phage. The difference may be due to the greater activity of the present preparation or *col<sub>1</sub>* phage may actually be found in much larger quantity than is the staphylococcus phage.

#### *General Properties of the Preparation*

*Composition*—The material obtained in this way has the composition of a nucleoprotein (Table II). It contains less than 1/10 per cent glucosamin and about 1 per cent glucose. The phosphorus content is higher than that reported by Schlesinger. There is a marked increase in amino nitrogen after acid hydrolysis. Hydrolyzed solutions give a precipitate with alkaline silver nitrate indicating the presence of purine bases.

The activity is destroyed by chymo trypsin but not by trypsin, by heating to  $50^{\circ}\text{C}$  for 5 minutes, by acidity greater than pH 5.0,

and by glycerine, alcohol, and acetone. It is most stable in solution in about 25 per cent saturated ammonium sulfate pH 7.0 but even under these conditions it loses activity at the rate of about 10 per cent a day at 10°C.

*Causation of Lysis by Purified Phage*—In the method used to titrate phage activity, lysis is not caused directly by the phage added but by the phage produced from this original quantity during the test. It seemed possible that several substances might be formed during this reaction and that the purified phage alone could not cause lysis. It was found, however, that addition of sufficient purified phage to

TABLE II  
*Analysis of Various Phage Preparations*

Preparation	C	H	N	P	Ash	NH <sub>2</sub> N	Glucose	Glucose amine
	per cent	per cent	per cent	per cent	per cent	per cent total N	per cent	per cent
Phage 7-10, precipitated cold 5 per cent trichloroacetic, washed free of ammonia N/100 hydrochloric acid. Dry alcohol—60°C—vacuum	40.6	5.4	14.6	4.6	13.0			
Phage 10-92	41.8	5.2	14.1	5.0			1.5	<0.1
Phage 68-7, precipitate with cold 5 per cent trichloroacetic acid, washed free of ammonia, dissolved N/10 sodium hydroxide						18.0		
Hydrolyze with 10 N hydrochloric acid, 110°C—1 hr						60.0	1.8	

bring the final phage concentration up to that present in a culture inoculated previously with a small amount of phage, caused immediate lysis. The result of such an experiment is shown in Fig. 1. In this figure 5 ml of a standard bacteria suspension was inoculated with 0.001 [ph u]/ml. At the end of 2 hours the phage concentration had increased to 10 [ph u]/ml and lysis began. 15 [ph u]/ml purified phage was then added to a control tube (B). Lysis began immediately showing that lysis may be caused by purified phage.

Dead bacteria, on the other hand, cannot be lysed by purified phage whereas crude phage solutions cause slow lysis. This confirms Bronfenbrenner's (14) results.

*Minimum Quantity of Phage Necessary to Produce Lysis*—Since growth of the bacteria is necessary for the production of phage it is evident that the minimum quantity of phage necessary to cause lysis depends on the concentration of the bacteria suspension used. It follows from the analysis of the reaction, described in a previous paper (15), that this effect disappears with very dilute bacteria suspensions and that under these conditions the quantity of phage required to produce lysis should be independent of the volume and concentration of the bacteria suspension.

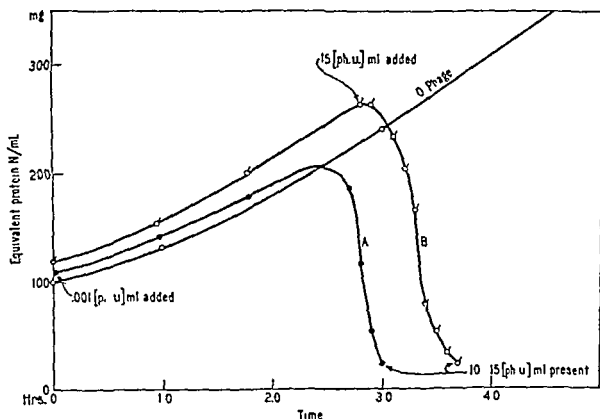


FIG. 1 Lysis caused by addition of purified concentrated phage

The results of an experiment in which a series of tubes of very dilute bacteria suspension (about 100–1000 bacteria/ml) were inoculated with decreasing quantities of purified phage are shown in Table III. From the number of such samples which show lysis it is possible by probability theory to calculate the average number of phage “units” per milliliter of the original solution on the assumption that one or more phage units will cause lysis under the conditions of the experiment. Tables for this calculation have been prepared by Halvorsen

and Ziegler (16) and the present results are calculated from their tables

When calculated in this way the final value of the quantity required to give positive results should be independent of the dilution if one particle can be detected and this is the result for phage titrations carried out by Feemster and Wells (17) The present experiments show a drift to smaller values of the "unit" as the dilution increases which would be the case if more than one particle were required to initiate the process The number of determinations is too small to be

TABLE III

*Minimum Quantity of Phage to Cause Lysis*

Phage solution diluted four times 5 500 with sterile normal saline, then diluted 10 100 four times with sterile broth Fresh bulb pipette used for each dilution Pipette rinsed five times in solution before removing sample 1 ml samples from each dilution added to series of 5 ml (or 500 ml) broth containing about 1000 staphylococci/ml, 37°C Number of tubes showing growth determined after 1, 2, 3 days

Protein N/ml added, mg	$1.5 \times 10^{-14}$	$3 \times 10^{-14}$				$3 \times 10^{-13}$				$3 \times 10^{-12}$	
Experiment	60	T	T	65	60	T	T	65		T	T
Volume culture, ml	5	5	500	5	5	5	500	5		5	500
No of samples	20	30	12	40	20	30	12	40		30	12
Fraction of samples containing no phage	0 95	0 83	0 84	0 75	0 55	0 40	0 42	0 25		0 17	0 18
Average number phage units/ml	0 05	0 18	0 18	0 28	0 6	0 9	0 9	1 4		1 7	1 7
Protein N/phage unit $10^{-14}$ mg	30	15	15	10	60	30	30	20		200	200

certain of the conclusion On the other hand it may be mentioned that Feemster and Wells added the bacteria to the phage suspension before sampling and since the phage is taken up by the bacteria it is possible that this method tests for the number of organisms which contain sufficient phage to detect rather than the number of phage particles themselves

The results of the present titrations show that about  $1 \times 10^{-13}$  mg protein nitrogen of purified phage will cause lysis This is equivalent to about  $5 \times 10^{-13}$  mg protein and if this quantity is assumed to be

one molecule then the gram molecular weight of the material is this number times Avogadro's number or  $5 \times 10^{-13} \times 6 \times 10^{23} = 30 \times 10^{10} \text{ mg} = 30 \times 10^7 \text{ gm}$ . This is about the same order of magnitude as found by Schlesinger (10) for the molecular weight by sedimentation velocity method in concentrated solution but is larger than that found by diffusion in dilute solution.

These results show that the presence of one or, at most, very few phage molecules (particles) can be detected. If this result is correct it is evidently impossible to account for the reactivation of inactivated phage by the assumption that inactivation was incomplete.

*Size of Phage Particle (Molecular Weight)*—Filtration experiments by Elford and Andrews (18) result in a calculated diameter for staphylococcus phage particles of about  $90 \text{ m}\mu (9 \times 10^{-6} \text{ cm})$ . This corresponds to a molecular volume of  $\frac{4}{3} \times \pi \times (5 \times 10^{-6})^3 \times 6 \times 10^{23}$  or  $36 \times 10^7 \text{ cm}^3$ . Bronfenbrenner and Muckenfuss found, however, that some active material passed through much smaller pores. Bronfenbrenner and Hetler (14) by the diffusion method also found a much smaller value corresponding to a radius of about  $1\text{--}10 \text{ m}\mu$  or a molecular weight of about  $10,000\text{--}800,000$ . Bronfenbrenner considered that in crude phage solutions part of the phage was combined with bacterial debris and that the phage particle itself was represented by the smaller figure.

Bronfenbrenner's diffusion experiments with crude phage were repeated and confirmed during the present work and it was expected that purified solutions would give constant values corresponding to the small particles. It was found, however, that concentrated purified phage solutions which contained  $0.1 \text{ mg}$  protein per ml or more gave very small diffusion values ( $D = \text{about } 0.001 \text{ cm}^2/\text{day}$ ) corresponding to a molecular weight of many million. If the diffusate from such experiments was replaced in the diffusion cell (19) and the diffusion repeated a constant value of about  $0.02 \text{ cm}^2/\text{day}$  was obtained corresponding to a molecular weight of about  $500,000$ . If the original solution is diluted to the corresponding concentration (about  $0.01\text{--}0.001 \text{ mg}$  protein/ml) the same value for the diffusion coefficient of  $0.02 \text{ cm}^2/\text{day}$  was obtained. It was further found that even concentrated phage solutions gave the same value for the first small



amount of phage which diffused out. These results confirm Bronfenbrenner's conclusion that there are various size phage particles but the smallest found in the present work had a diffusion coefficient of about  $0.02 \text{ cm}^2/\text{day}$ . They indicate, in addition, that the small and large particles are in equilibrium since by simple dilution the large value for the diffusion coefficient is obtained.

TABLE IV

*Diffusion Coefficient*

Diffusion method of Anson and Northrop— $10^\circ\text{C}$

Inside solution—bacteriophage dissolved in 0.20 saturated ammonium sulfate  
pH 7.0, 45 ml

Outside solution—0.16 saturated ammonium sulfate pH 7.0, 15 ml

Experiment No	Time	Diffusion coefficient $\text{cm}^2/\text{day}$	
		By phage activity	By protein nitrogen
	<i>days</i>		
1	3	0.010	0.017
	6	0.035	0.016
2	7	0.015	0.026
3	3	0.017	0.017
	6	0.037	0.020
4	6	0.016	0.030
	Average	$0.022 \pm 0.004$	$0.021 \pm 0.004$
5 Outside solution from Experiment 4		0.019	

Average  $D = 0.021 \text{ cm}^2/\text{day}$

$$r = \frac{RT}{DN} = \frac{1.17 \times 10^{-8} \text{ cm}}{0.021} = 5.6 \times 10^{-7} \text{ cm}$$

$R = 8.3 \times 10^7 \text{ ergs degree}^{-1} \text{ mol}^{-1}$ ,  $N = 6.06 \times 10^{23} \text{ mol}^{-1}$ ,  $T = 283 \text{ degrees}$ ,  
 $\eta = 0.015 \text{ ergs sec cm}^{-3}$

Mol volume  $= 4/3\pi r^3 N = 450,000 \text{ cm}^3$

Assumed density  $= 1.3$

Mol weight  $= 580,000$

$$1 \text{ molecule} = \frac{5.8 \times 10^8 \text{ mg}}{6 \times 10^{23}} = 1 \times 10^{-15} \text{ mg} \quad 1 \times 10^{-15} \text{ mg N (approximate)}$$

A summary of several experiments in which the diffusion coefficient was determined by both protein nitrogen and phage activity is shown in Table IV. It is evident that the protein nitrogen and the activity diffuse at the same rate.

*Rate of Sedimentation in Ultracentrifuge*—Svedberg (20) has shown that the molecular weight may be calculated from the rate of sedimentation in a centrifugal field. Schlesinger (10), by this method, had calculated a diameter of about 90  $\mu$  agreeing with the filtration figure but this result was obtained with concentrated solutions. Gratia (21) obtained smaller values.

The sedimentation velocity of purified phage has been measured by Wyckoff (22) who found the preparation to be practically homogeneous in size and to have a sedimentation content of  $650 \times 10^{-13}$  cm dyne<sup>-1</sup> sec<sup>-1</sup> corresponding to a molecular weight of hundreds of millions. This determination again was necessarily done in concentrated solution.

The sedimentation constant unfortunately cannot be determined by the centrifuge method in the range of concentration ( $<0.01$  mg/ml) in which the diffusion method shows the phage to be of much smaller size. It is possible, however, to test qualitatively the effect of dilution by centrifuging solutions of different concentrations in relatively large tubes and determining the phage concentration in the top and bottom half of the tubes. If the active particles are all of the same size, or if they are a mixture of several different sizes which are independent of each other, then it would be expected that the ratio of the concentration in the bottom half to that in the top would be constant at all concentrations, while if there were large particles which dissociated into small ones on dilution then the ratio of the bottom to top would decrease as the solution was diluted. The results of such an experiment are shown in Table V. All tubes of any one experiment were centrifuged together in the same head. A tube containing tobacco mosaic virus protein, which is known to have a molecular weight of 17 million (Stanley (8)) is included for comparison. The results show that the more dilute the solution the smaller the fraction of the phage which is found in the bottom half of the tube. They also indicate an equilibrium between large and small particles since the bottom portion of a solution which has been sedimented behaves upon dilution just as the top half of the same solution when diluted to the same concentration. They show further that in dilute solution the phage particle sediments more slowly than does tobacco mosaic (molecular weight 17,000,000) while in concentrated solution it sediments more rapidly.

The results as a whole indicate that phage exists in solution in varying sized particles with a maximum diameter of about  $1 \times 10^{-5}$  cm (100 m $\mu$ ) corresponding to a molecular volume of about

TABLE V

*Effect of Concentration on Rate of Sedimentation of Phage Solutions*

Solution in 10 cc cups in Wyckoff air centrifuge, 20 min at 40,000  $\times$  gravity  
Top half pipetted off and phage concentration determined in top and bottom half of solution

Solvent		0.25 saturated ammonium sulfate N/20 sodium phosphate pH about 7.5 sp gr 1.10									
Original phage conc [ph u] ml = phage protein N $\gamma$ /ml		300 60	100 20	30 6	10 2	3 0.6	1 0.2	0.3 0.06	0.1 0.02	0.03 0.006	0.01 0.002
Exp No	Solution	Ratio bottom half/top half									
22	Bottom solution from 22	40		15		6		2.5			
23				14			6				
24		30	30	17		6		3.5			
26	Crude phage						3.5				
	Top solution from 24						4.5				
	Bottom solution from 24						3.0				
Solution		Sp gr 1.03 40,000 $\times$ gravity 15 min half Ratio bottom half/top half									
11	M/10 phosphate pH 7.4										
	0.05 sat ammonium sulfate				350	140	75				
39	Crude phage solution				40						
	Crude phage solution pH 8.5						3				
40	Crude phage diluted with N/20 sodium phosphate pH 7.4 plus inactive phage				400	300		250		100	
43	" " " "				350			100		30	
	Crude phage diluted with N/20 sodium phosphate pH 8.0							4			4
39	Tobacco mosaic					5-10-15					

300,000,000 cm<sup>3</sup> and a minimum diameter of about  $1 \times 10^{-6}$  cm corresponding to a molecular volume of about 500,000 cm<sup>3</sup>

When the solution is diluted with an inactivated portion of the same

solution the effect of dilution on the sedimentation rate is less marked. Measurements of such inactive solutions, by the usual ultracentrifuge technique show that they contain only small molecules with a sedimentation constant corresponding to a molecular weight of about 500,000 which is the same as that found for the active particles in high dilution. If the small inactive particles could enter into the equilibrium with the active ones the effect would be to decrease dissociation on dilution.

No loss in activity occurred during these experiments.

Svedberg (20) and his collaborators have found that some proteins of high molecular weight, especially certain hemocyanines, also dissociate into smaller molecules. In this case the small molecules appear even in concentrated solution in alkaline pH ranges. Wyckoff (22) has found that some virus proteins also dissociate in alkaline solution. It is evident, however, that if the various size particles are in equilibrium in alkaline solution they must be in equilibrium at all ranges of acidity although the percentage of the smaller ones present may be extremely small. However, if the solutions were infinitely dilute the protein would be present as small molecules at all pH. This appears to be the case with bacteriophage.

*Activity of Small and Large Phage Molecules* —The preceding experiments indicate that phage is present in solution in the form of large and small molecules which are in equilibrium with each other. Since the large molecules increase as the concentration increases, the activity of the solution would increase faster than the total concentration, if the large molecules alone were active, and more slowly if the small molecules alone were active. Actually, it is found that the time required to cause lysis is inversely proportional to the log of the phage concentration instead of to the concentration itself as is usually the case with enzyme reactions. This result, however, can be derived (15) from the peculiar relation between bacteria growth and phage production and therefore does not necessarily mean that the phage activity increases more slowly than the concentration. It must be remembered also that the activity measurements are all carried out in dilute solution ( $< 1\gamma/\text{ml}$ ) in which range the phage is present practically entirely in the form of small molecules. The question as to the relative activity of the large and small molecules cannot therefore be definitely decided at present.

*Relation of Phage Activity to the Protein*—If the nucleoprotein is really the active agent it would be expected that denaturation or digestion of the protein would result in corresponding loss in activity. The protein is instantly inactivated in solution more acid than pH 4.7. No visible precipitate forms, even in the presence of salt, but the solution becomes more viscous and the denatured protein may be filtered out. Measurement of the sedimentation constant shows the

TABLE VI  
*Inactivation and Denaturation at Various pH*

Noted amount M/1 acetic acid added to 5 ml phage solution in M/10 pH 7.0 phosphate buffer,  $\frac{1}{4}$  saturated ammonium sulfate. Solution at 20°C for 5 minutes, titrated back to pH 7.0 by 1 M ammonium hydroxide. 0°C for 24 hrs. Filtered at 0°C through No. 42 paper. Samples removed and analyzed for ph. u. and protein N as noted.

Ml M/1 acetic acid	0			0.7			1.0		
pH (colorimetric)	7.0			4.7			4.4		
	[ph. u.] ml	[protein N] ml	[ph. u.] mg protein N	[ph. u.] ml	[protein N] ml	[ph. u.] mg protein N	[ph. u.] ml	[protein N] ml	[ph. u.] mg protein N
Solution analyzed immediately after neutralization, No. 1	500	0.08	6300	500	0.08	6300	1.0	0.08	1.0
After 24 hrs. 0°C, No. 2	500	0.08	6300	500	0.08	6300	1.0	0.08	1.0
Solution No. 2, Filter No. 42	500	0.07	6600	420	0.06	7000	1.0	<0.001	>1000
2 ml solution No. 2 plus 4 ml $\frac{1}{4}$ saturated ammonium sulfate—viscosity measured 25°C									
$\frac{\eta \text{ sol}}{\eta \text{ H}_2\text{O}}$			1.3			1.3			1.55

denatured protein to be of much smaller molecular weight than the active protein.

The result of an experiment in which the protein was denatured by acid is shown in Table VI. At pH 7.0 and 4.7 there is no change in activity in 24 hours. At pH 4.4 practically all the activity is lost after 5 minutes at 20°C, the total protein content remains constant but the viscosity increases and after standing at 0°C for 24 hours the denatured, inactive protein may be filtered off.

*Inactivation and Denaturation at Various Temperatures*—The result of an experiment in which solutions at pH 7.0 were heated varying lengths of time at different temperatures is shown in Table VII. The loss in activity is again accompanied by the formation of denatured protein. It may be noted that the specific activity ( $[\text{ph u}]/\text{mg protein N}$ ) in this experiment is low throughout and at 55°C tends to decrease. This indicates that the preparation already contained some denatured protein and also that the separation of native and

TABLE VII

*Inactivation and Denaturation of Phage Protein Solution pH 7.0 at Various Temperatures*

Solution in  $\text{M}/10$  pH 7.0 phosphate,  $\frac{1}{4}$  saturated ammonium sulfate left at temperatures shown and samples removed and cooled to 0°C for 24 hrs after time interval shown. Samples filtered through No. 42 paper at 0°C and activity and protein nitrogen determined on filtrate.

Temperature.	55 C.			37 C.			25 C.			0 C.		
Time												
Hrs	$[\text{ph u}]/\text{ml}$	$[\text{protein N}]/\text{ml}$	$[\text{ph u}]/\text{mg protein N}$	$[\text{ph u}]/\text{ml}$	$[\text{protein N}]/\text{ml}$	$[\text{ph u}]/\text{mg protein N}$	$[\text{ph u}]/\text{ml}$	$[\text{protein N}]/\text{ml}$	$[\text{ph u}]/\text{mg protein N}$	$[\text{ph u}]/\text{ml}$	$[\text{protein N}]/\text{ml}$	$[\text{ph u}]/\text{mg protein N}$
0	20	0.01	2000	50	0.015	3500	50	0.015	3500	50	0.015	3500
0.016	10	0.007	1400									
0.03	5	0.005	1000									
0.16	0.1	0.001										
24.0				2	0.001	2000	15	0.005	3000			
72.0							5	0.001	5000			
120.0										20	0.007	3000
480.0										2	0.001	2000

denatured protein is not quantitative. This effect was also noted in analyzing known mixtures of native and denatured protein and renders this type of experiment less conclusive.

Krueger and Mundell (23) have shown that such heat inactivated phage may be reactivated under certain conditions. Lominski (24) has also reported similar results. This is probably a case of the reversal of denaturation of a protein and may furnish the explanation for Kendall's (25) experiments in which it was found that active phage may be obtained from autoclaved solutions.

*Hydrolysis of Phage by Various Enzymes*—The literature concerning the digestion or inactivation of phage by enzymes, especially "trypsin," is controversial. This is probably due to the fact that crude trypsin preparations, which contain several enzymes, have been used. It was found in the present work that phage was inactivated by chymo-trypsin (26) but not by trypsin. The denatured protein is not hydrolyzed by either pepsin, trypsin, or chymo trypsin. The effect of these enzymes on active and inactive phage preparations is shown in Table VIII. It will be noted that there is no significant change in the concentration of total protein nitrogen. There is, however, a qualitative change in the protein when the activity is destroyed by chymo trypsin. After inactivation the protein has the same properties as protein denatured by heat and may be removed by filtration or centrifugation. There is in this case also an increase in viscosity and concentrated solutions set to a gel. This action of chymo trypsin on the bacteriophage therefore appears to be very similar to its action on casein (26). Dilute chymo trypsin causes milk or casein solutions to clot without any marked change in total protein concentration although more concentrated enzyme solutions do digest the protein. The concentration of chymo trypsin required to inactivate phage solutions is very much higher than that required to clot milk and it is probable that still higher concentrations would hydrolyze the protein but the accurate determination of phage protein in the presence of such large amounts of chymo trypsin is experimentally difficult.

The results of a series of experiments in which the loss in phage activity was compared with the denatured protein formed is shown in Table IX. The rate of inactivation of the phage is proportional to the concentration of chymo trypsin, indicating a true catalytic reaction. Slightly more protein was denatured than corresponded to the loss in activity so that the protein remaining was slightly more active than the original but the difference is hardly outside the experimental error.

*Sedimentation of Phage Activity and of the Protein in the Ultracentrifuge*—It was stated above that the preparation was found to be homogeneous when examined in the analytical centrifuge. In the absence of other evidence, however, this result cannot be considered

The results as a whole indicate that phage exists in solution in varying sized particles with a maximum diameter of about  $1 \times 10^{-5}$  cm (100 m $\mu$ ) corresponding to a molecular volume of about

TABLE V

*Effect of Concentration on Rate of Sedimentation of Phage Solutions*

Solution in 10 cc cups in Wyckoff air centrifuge, 20 min at 40,000  $\times$  gravity  
Top half pipetted off and phage concentration determined in top and bottom half of solution

Solvent.		0.25 saturated ammonium sulfate N/20 sodium phosphate pH about 7.5 sp gr 1.10									
Original phage conc. [ph u] ml = phage protein N $\gamma$ /ml		300 60	100 20	30 6	10 2	3 0.6	1 0.2	0.3 0.06	0.1 0.02	0.03 0.006	0.01 0.002
Exp No	Solution	Ratio bottom half/top half									
22	Bottom solution from 22	40		15		6		2.5			
23				14			6				
24		30	30	17		6		3.5			
26	Crude phage						3.5				
	Top solution from 24						4.5				
	Bottom solution from 24						3.0				
Solution		Sp gr 1.03 40,000 $\times$ gravity 15 min half Ratio bottom half/top									
11	{ M/10 phosphate pH 7.4 0.05 sat ammonium sulfate				350	140	75				
39					40						
	Crude phage solution										
	Crude phage solution pH 8.5						3				
40	Crude phage diluted with N/20 sodium phosphate pH 7.4 plus inactive phage				400	300		250		100	
43	" " " "				350			100		30	
	Crude phage diluted with N/20 sodium phosphate pH 8.0							4			4
39	Tobacco mosaic					5-10-15					

300,000,000 cm<sup>3</sup> and a minimum diameter of about  $1 \times 10^{-6}$  cm corresponding to a molecular volume of about 500,000 cm<sup>3</sup>

When the solution is diluted with an inactivated portion of the same



solution the effect of dilution on the sedimentation rate is less marked. Measurements of such inactive solutions, by the usual ultracentrifuge technique show that they contain only small molecules with a sedimentation constant corresponding to a molecular weight of about 500,000 which is the same as that found for the active particles in high dilution. If the small inactive particles could enter into the equilibrium with the active ones the effect would be to decrease dissociation on dilution.

No loss in activity occurred during these experiments.

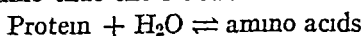
Svedberg (20) and his collaborators have found that some proteins of high molecular weight, especially certain hemocyanines, also dissociate into smaller molecules. In this case the small molecules appear even in concentrated solution in alkaline pH ranges. Wyckoff (22) has found that some virus proteins also dissociate in alkaline solution. It is evident, however, that if the various size particles are in equilibrium in alkaline solution they must be in equilibrium at all ranges of acidity although the percentage of the smaller ones present may be extremely small. However, if the solutions were infinitely dilute the protein would be present as small molecules at all pH. This appears to be the case with bacteriophage.

*Activity of Small and Large Phage Molecules* —The preceding experiments indicate that phage is present in solution in the form of large and small molecules which are in equilibrium with each other. Since the large molecules increase as the concentration increases, the activity of the solution would increase faster than the total concentration, if the large molecules alone were active, and more slowly if the small molecules alone were active. Actually, it is found that the time required to cause lysis is inversely proportional to the log of the phage concentration instead of to the concentration itself as is usually the case with enzyme reactions. This result, however, can be derived (15) from the peculiar relation between bacteria growth and phage production and therefore does not necessarily mean that the phage activity increases more slowly than the concentration. It must be remembered also that the activity measurements are all carried out in dilute solution ( $< 1\gamma/\text{ml}$ ) in which range the phage is present practically entirely in the form of small molecules. The question as to the relative activity of the large and small molecules cannot therefore be definitely decided at present.

is gradually transformed to the active enzyme, trypsin (31) This reaction is autocatalytic just as is the rate of increase of bacteriophage Similarly, an inert protein has been isolated from gastric mucosa which, when inoculated with pepsin under proper conditions, is transformed into active pepsin (32) The reaction is again autocatalytic The immunological relations have been worked out in the latter case and found to be similar to those of the bacteriophage or tobacco mosaic systems (39) The pepsin is immunologically distinct from the other proteins of the animal and also from its inactive precursor The enzyme and its precursor are, in an immunological sense, foreign proteins as is the protein of the lens or the bacteriophage or tobacco mosaic protein A further analogy with the phage and the viruses consists in the fact that pepsin of one species may be "adapted" to another species since if swine pepsinogen is inoculated with chicken pepsin, swine pepsin is produced, and not the chicken pepsin used for inoculation (33)

It will be noted that, although other enzymes may be "grown" by an autocatalytic reaction in solutions of their precursors, this reaction is not synthetic but is probably an hydrolysis It is extremely improbable that any protein can be synthesized by a purely catalytic reaction under biological conditions since it is known that under such conditions of temperature and pH proteins hydrolyze slowly into their constituent amino acids and this process may be accelerated by a number of catalysts If, now, another catalyst could be found which would cause the synthesis of proteins from amino acids without the expenditure of energy, a perpetual motion would result<sup>2</sup> There is

<sup>2</sup> It is possible to assume that the reaction



is reversible but that the concentration of protein at equilibrium is extremely small Even though only traces of protein were present, still large quantities could be formed by this reaction if a mechanism existed to remove the protein from the solution If the protein were very insoluble it would be continually removed as it was formed and in this way the total amount formed might be large This may be the explanation of "plastein formation" since the "protein" formed is insoluble and since the reaction does not occur with proteins like gelatin which do not form insoluble precipitates in the presence of pepsin

From this point of view the formation of the viruses and bacteriophages might be accounted for by the assumption that they are polymers of smaller proteins

good reason to believe, therefore, that the synthesis of protein requires energy and that it can only take place in a system organized to supply this energy, as in a living cell. Thus, the cells of the gastric mucosa synthesize the inert protein, pepsinogen, which is transformed to the active enzyme by an autocatalytic reaction.

A similar mechanism will evidently account for the increase of bacteriophage and other viruses in the presence of living cells. The cells synthesize a "normal" inactive protein. When the active virus or bacteriophage is added, this inactive protein or "pro phage" is transformed by an autocatalytic reaction into more active phage. This mechanism accounts for the fact that phage is produced rapidly only in the presence of growing cells since only in growing cells is synthesis taking place. Evidently the amount of "pro phage" present in the cells at any one time must be small and hence the failure to obtain large increases of the active agent in the absence of living cells is to be expected. It might be expected that under certain conditions enough of the inert protein would be present to cause a detectable increase in phage without growth of cells and such a case has recently been reported by Krueger (34). Small increases in phage under these conditions have also been reported by Gratia (35). If the "pro phage" present in the cells is kept constant while growth occurs the rate of formation of the active material will be proportional only to the amount already present, *i.e.*

$$\frac{dP}{dt} = KP$$

and this is the experimental result. Since the rate of the phage reaction is faster than that of bacterial growth phage will sooner or later be formed faster than the "pro phage" and the rate will then depend on the growth rate of the bacteria. It is possible that lysis occurs at this point.

The active protein is of much higher molecular weight than most

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and that the process of polymerization continually removes the original small protein molecule from the system. It would still be necessary to assume that the small units were formed autocatalytically from the constituent amino acids. There appears to be no evidence for this reaction and the whole mechanism is more complicated than that outlined above.

normal proteins and this is true of viruses in general Svedberg (20) and his collaborators have found, however, that some "normal" proteins are nearly as large and further that these large molecules dissociate under certain conditions into smaller molecules of the same order of size as "normal" proteins This process appears to be reversible so that there is an equilibrium between large and small protein molecules The formation of very large protein molecules from small ones, therefore, is quite different from the formation of protein from amino acids since it may go in either direction without the necessity of added energy The large active protein molecule could, therefore, be formed from a small inactive protein molecule without energy being added and it is not necessary to assume that this inactive precursor is of very high molecular weight It was shown above that there is reason to believe that phage itself exists in small and large molecules which are in equilibrium with each other

It appears to the writer that the assumption that the living host cells synthesize an inert "normal" protein which is changed to the active phage by an autocatalytic reaction accounts for the observed facts as well as does the far more complicated series of assumptions involved in the hypothesis that the phage itself is a living organism

### *Experimental Methods*

*Protein Nitrogen Determination*—1 ml of solution is added to 9 ml 5 per cent trichloroacetic acid and the suspension allowed to stand at least 15 minutes at 20°C Turbidity compared in Klett photoelectric colorimeter (36) with standard suspension, the protein nitrogen content of which had been determined by Kjeldahl method In the range of from 0.5 to 5% protein nitrogen/ml the colorimeter reading is inversely proportional to the concentration of the suspension For convenience a standard suspension may be read against M/25 copper sulfate made up in M/10 sulfuric acid and a curve plotted This curve may then be used for analyzing unknown solutions against the copper sulfate The colorimeter readings are accurate to less than 5 per cent but if the protein differs qualitatively from that used as a standard the determination may be in error by 50 per cent or more It is therefore necessary to check determinations occasionally by the Kjeldahl method, especially during the purification process

*Phage Activity*—The method used was a slight modification of that described by Krueger (13) More concentrated bacteria suspension and more concentrated phage solutions were used in order to shorten the time of the measurement, to avoid trouble due to contaminating organisms, and to obviate long serial dilutions

*Standard Staphylococci Suspension*—Growth from 18 hour Blake bottles is washed off with sterile broth and the suspension diluted with broth so as to contain  $0.3 \text{ mm}^3$  bacteria/ml as determined by centrifuging method (37). This is equivalent to 0.03 mg protein nitrogen/ml. if the bacteria suspension is standardized in the colorimeter against a casein solution or to about  $1 \times 10^8$  organisms/ml by plate count. 5 ml. of this standard suspension is pipetted into a series of  $20 \times 15 \text{ cm}$  sterile test tubes. 1 ml. of the phage solution to be titrated is added to one tube, 0.6 ml. of this suspension is added to the next tube, 0.56 of this to the third, etc. In this way a series of tubes is prepared containing 1.0, 0.1, 0.01, 0.001 ml., etc. of the phage solution. The tubes are placed in a shaker in the water bath at  $36^\circ\text{C}$  and the time determined at which the turbidity of the tubes matches that of a standard tube containing 0.05 mg protein nitrogen/ml equivalent. The relation of the time of lysis during the log phase of growth to the quantity of phage activity is expressed by an equation of the general form

$$t = K \log P + C$$

If one phage unit is defined as the quantity of active phage which will cause lysis of 5 ml. of standard bacteria suspension in 1 hour then the equation becomes

$$t = K \log P + 1$$

The value of  $K$  (which is the slope of the line when  $\log P$  is plotted against time for lysis) depends on the rate of growth of the culture so that anything which affects the rate of growth affects the determination. It is necessary therefore, to use the same broth and, if possible, run the determination simultaneously if strictly comparable results are to be obtained. Under these conditions an error of less than 5 per cent may be obtained. Determinations made at different times with different culture media and different bacteria suspensions may differ by many times. For this reason a series of dilutions of a crude standard phage which was kept at  $0^\circ\text{C}$  was run with each set of unknown solutions. If the value for the standard phage differed significantly from the average value obtained in a series of measurements the determination was discarded.

Under the conditions used in the present work the value of  $K$  was 0.6 hour and the equation used therefore was

$$-\log_{10} P = \frac{t - 1}{0.60}$$

Solving for  $P$  this gives the following values

$T$ (hours)	1	0	1	1	2	3	4	5	6	7						
$P$ (phage units)	1	0	0	7	0	5	0	32	0	22	0	15	0	10	0	07

The relation holds only during the logarithmic phase of bacteria growth which under the present conditions was from 1 hour to 3 hours

35,000 liters of crude phage solution have been prepared during the course of these experiments and many thousand preparations analyzed for phage activity and protein nitrogen. This part of the work was carried out by Miss Elizabeth Shears to whom the writer is also indebted for assistance in the preparation of the manuscript.

#### SUMMARY

1 A method for isolating a nucleoprotein from lysed staphylococci culture is described.

2 It is homogeneous in the ultracentrifuge and has a sedimentation constant of  $650 \times 10^{-13}$  cm dyne<sup>-1</sup> sec<sup>-1</sup>, corresponding to a molecular weight of about 300,000,000.

3 The diffusion coefficient varies from about 0.001 cm<sup>2</sup>/day in solutions containing more than 0.1 mg protein/ml to 0.02 in solutions containing less than 0.001 mg protein/ml. The rate of sedimentation also decreases as the concentration decreases. It is suggested, therefore, that this protein exists in various sized molecules of from 500,000–300,000,000 molecular weight, the proportion of small molecules increasing as the concentration decreases.

4 This protein is very unstable and is denatured by acidity greater than pH 5.0, by temperature over 50°C for 5 minutes. It is digested by chymo-trypsin but not by trypsin.

5 The loss in activity by heat, acid, and chymo-trypsin digestion is roughly proportional to the amount of denatured protein formed under these conditions.

6 The rate of diffusion of the protein is the same as that of the active agent.

7 The rate of sedimentation of the protein is the same as that of the active agent.

8 The loss in activity when susceptible living or dead bacteria are added to a solution of the protein is proportional to the loss in protein from the solution. Non-susceptible bacteria remove neither protein nor activity.

9 The relative ultraviolet light absorption, as determined directly, agrees with that calculated from Gates' inactivation experiments in the range of 2500–3000 Å u but is somewhat greater in the range of 2000–2500 Å u.

10 Solubility determinations showed that most of the preparations contained at least two proteins, one being probably the denatured form of the other. Two preparations were obtained, however, which had about twice the specific activity of the earlier ones and which gave a solubility curve approximating that of a pure substance.

11 It is suggested that the formation of phage may be more simply explained by analogy with the autocatalytic formation of pepsin and trypsin than by analogy with the far more complicated system of living organisms.

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# AN ULTRACENTRIFUGAL ANALYSIS OF CONCENTRATED STAPHYLOCOCCUS BACTERIOPHAGE PREPARATIONS

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PLATES 2 AND 3

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During the later stages of Northrop's study (1) of the bacteriophage for *S. albus*, the writer has had the opportunity of examining many of his preparations with an analytical ultracentrifuge arranged for ultraviolet absorption measurements according to the original method of Svedberg (2). Several things can be learned from the sedimentation diagrams of dilute protein solutions photographed in this way—(1) the rate of sedimentation of any heavy molecules that may be present, (2) the homogeneity, or uniformity with respect to size and shape, of these molecules or particles, and (3) the purity of the solution. The results of the present paper throw light upon each of these points but it is clear that many of these bacteriophage solutions have a complexity that can only be resolved by much more detailed investigation.

The instrument used in these experiments was a development of the air driven machine already described (3). The ultraviolet light, of wave lengths between 2700Å and 2300Å, was that of a high pressure mercury arc passed through the usual chlorine and bromine filters. In all experiments the photography, which was carried out automatically (4), was such that the interval between pictures was exactly 5 minutes and the exposure  $1\frac{1}{2}$  seconds. Preparations of concentrated staphylococcus bacteriophage have unusually strong absorption in the ultraviolet region—for a 2 mm thick cell the optimum concentration has been ca 0.3 mg per cc.

The bacteriophage solutions that have been photographed fall into two main groups—final products of the chemical purification possessing the greatest concentration of bacteriophage activity thus far

obtained, and such preparations inactivated by alkali, by chymotrypsin, and by heat. All the very active solutions gave sedimentation pictures like those in Figs 1-5. In general these solutions contain more than one ultraviolet-absorbing constituent. They always have shown relatively sharp, rapidly sedimenting boundaries like those of Figs 1 and 5 but there also have been present larger or smaller amounts of an absorbing material that does not sediment to a measurable extent after 1 hour in a field of 200,000 gravity and sometimes traces have been seen of a "gel" structure like that to be described for heat-inactivated solutions. The factors which determine the amount and the production of these light contaminants are as yet very incompletely understood. Experiment (1) shows that all but a trace of the bacteriophage activity is precipitated from a solution held for an hour at 40,000 gravity, this demonstrates that it cannot be a property of the light "unsedimentable" material. In view of the comparative ease with which it is centrifuged from these concentrated solutions, an association of the bacteriophage with the sharp, rapidly sedimenting boundary seems probable. Though this boundary is always the most conspicuous feature in diagrams made from the final purified solutions, the amount of the substance producing it is not simply proportional to the activity. Thus, practically inactive solutions have given photographs indistinguishable from Fig 1, similarly the solution yielding Figs 4 and 5 was four times as active as that of Fig 1 though their contents of protein nitrogen were the same. Assuming that a connection really exists between the heavy boundary and the bacteriophage, this must mean that the bacteriophage molecule or particle may lose activity without a detectable alteration in shape or size.

The sedimentation constants of the heavy substance measured in different active preparations have varied from about  $550$  to  $670 \times 10^{-13}$  cm sec<sup>-1</sup> dynes<sup>-1</sup>. Of these the upper value is probably near the true one. There are two reasons for the lower constants that have been found. The more important one is the presence of "unsedimentable" material and of small amounts of gel that raise the viscosity of the medium through which the heavy particles are moving. An attempt was made to wash these light impurities away by throwing down the heavy substance in the quantity ultracentrifuge. This was unsuc-

cessful because some of the activity of the bacteriophage seems to be lost by one ultracentrifugal precipitation and nearly all is destroyed by repetition of the procedure. The seemingly low constants found in certain samples were due to the presence of ammonium sulfate which raised the density of the solvent slightly above unity. Because the density of the bacteriophage itself appears to be lower than that of most proteins, it takes relatively little ammonium sulfate to cause a considerable reduction in the rate of sedimentation.

Determinations of density have been possible from comparisons of the sedimentation rates in water and in quarter saturated ammonium sulfate. Several years ago Bechhold (5) found for *B. coli* bacteriophage a density of 1.10 and considered this evidence for its microbic character, the mere fact that staphylococcus bacteriophage is readily sedimented from its concentrated solutions in quarter saturated ammonium sulfate shows that its density cannot be so low. Two agreeing estimates of the density of the heavy component have been made. One preparation was made up in water and in a salt solution having a density of 1.10. In water the sedimentation constant  $s_{20}^w$  was  $646 \times 10^{-13}$ , in the salt it was  $s_{20} = 320$ . These rates are connected by the relation

$$\frac{s_{20}^w}{s_{20}} = \frac{1 - V\rho_w}{1 - V\rho_s}$$

where  $1/V$  is the density of the sedimenting heavy particles. Substituting the above constants,  $V$  becomes 0.834 and  $1/V = 1.20$ . In another experiment in which the solvent had  $\rho = 1.12$ ,  $s_{20}^w$  was 240 (Fig. 4), the same preparation diluted with water until  $\rho = 1.04$  gave  $s_{20} = 495$  (Fig. 5). The density of the particles producing these boundaries calculates out to be 1.19, a value decidedly lower than the 1.33 characterizing most simple proteins.

Besides retarding the rate of sedimentation through increasing the density of the solvent, the presence of considerable amounts of ammonium sulfate leads to a pronounced diffuseness of boundary (Fig. 4). The reason for this is not yet clear.

In one or two instances a series of very faint sharp boundaries (cf. Fig. 3) was observed sedimenting at about a third the rate of the heavy component. The substance responsible for these has not been

identified but there seems to be no reason for relating it intimately to the bacteriophage activity

The molecular weight of the heavy component cannot be calculated from its sedimentation constant until the diffusion constant is known, but it is possible to establish a lower limit for this weight. According to Svedberg (6)

$$M = \frac{RTs_{20}^0}{D(1 - \bar{V}\rho)}$$

where  $M$  is the molecular weight,  $R$  the gas constant,  $T$  the absolute temperature, and  $D$  the diffusion constant. Taking  $1/\bar{V} = 1.20$  and  $s_{20}^0 = 650 \times 10^{-13}$ ,  $M = 9.60/D$ . If one examines the diffusion constants determined for very large molecules by Svedberg (6) and his students, it is evident that  $D$  can scarcely be less than  $0.5 \times 10^{-7}$ . On this basis the heavy component of active bacteriophage solutions would have a molecular weight of at least 200 millions.

The heavy component is not split when concentrated bacteriophage solutions are inactivated by chymo-trypsin. In every instance, however, the boundaries after inactivation were more diffuse (Fig. 10). The sedimentation constants also have been low, around 550–600, but, since all these inactive preparations have contained an especially large amount of unsedimentable matter, it is impossible to say whether chymo-trypsin inactivation has led to small changes in the shape or size of the molecules of the heavy substance.

Bacteriophage solutions at pH 10 gradually become inactive over a period of several days. Photographs of such solutions have been made immediately after alkalinization and after 3 days. As can be seen from Fig. 2 the heavy boundary becomes fainter while at the same time there is an increase in the amount of unsedimentable material.

Inactivation by heating for 10 minutes at a temperature of 60°C leads to a sedimentation diagram different from any of the foregoing. The most conspicuous feature of this new diagram is an extraordinarily sharp boundary that moves outward in the cell only under the influence of intense fields. At first sight this might seem due to large molecules of a very low density but its behavior in quarter-saturated ammonium sulfate and in very dilute salt solutions conflicts with such an interpretation. Instead there are reasons for considering that it

arises through the compression of a dilute protein gel. The abnormally high viscosities of these dilute inactive solutions point in this direction but the best evidence for a gel structure is the variation in observed sedimentation constants from different preparations. They have been as low as  $9 \times 10^{-12}$  and as high as  $23 \times 10^{-12}$ . If the protein is sedimented only part way down in the analytical cell, the preparation can be rehomogenized by mixing. When this is done, however, the new boundaries formed during a second centrifugation are ordinarily less sharp (Fig 7) and move more rapidly than before. The remixed gel is apparently less stable than it was before ultracentrifugation because its structure often collapses and the boundary disappears during the course of a second centrifugation. Thus one preparation on its first partial sedimentation (Fig 8) gave  $s_0 = 10$ , after remixing it yielded a boundary corresponding to  $s_{20} = 22$  which vanished after the first four or five pictures (Fig 9). Heat inactivated solutions commonly contain in addition to this gel an ultraviolet absorbing substance which sediments still more slowly with a diffuse boundary (e of Figs 8 and 9) corresponding to  $s_{20} \approx ca\ 3 \times 10^{-12}$ . Its occurrence suggests that it is one of the products resulting from thermal destruction of the large particles with  $s_{20} = 650 \times 10^{-12}$ .

The writer is indebted to J H Northrop for the solutions studied and for many helpful discussions of their properties.

#### SUMMARY

Analytical observations have been made with the air ultracentrifuge on concentrated staphylococcus bacteriophage solutions and on these solutions inactivated by alkali, chymo trypsin, and heat.

All active solutions contain a homogeneous heavy component that sediments with a constant of  $s_{20} = ca\ 650 \times 10^{-12}$  cm sec<sup>-1</sup> dynes<sup>-1</sup>, has an apparent density of  $ca\ 1.20$ , and a molecular weight probably not less than 200 millions. There is also present some very light ultraviolet absorbing material which is not a carrier of bacteriophage activity. The amount of the heavy component is not strictly proportional to the bacteriophage activity so that if the activity resides in it, as appears to be the case, inactivation may occur without measurable change in molecular size and shape.

When the bacteriophage solutions are inactivated by chymo trypsin

the heavy component is not disrupted but the sedimenting boundary have always been fairly diffuse. As the activity gradually diffuses from alkaline solutions, the heavy component is replaced by unsedimentable material. When a solution is inactivated by heat, a dilute gel is produced which sediments with an exceptionally sharp boundary in a relatively intense centrifugal field.

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## EXPLANATION OF PLATES

## PLATE 2

FIG 1 A series of sedimentation photographs of an active bacteriophage preparation. The boundaries marked (b) are due to the heavy component. The meniscus (a) separates the air-bubble above from the solution below. This preparation contained less unsedimentable material (which shows the greater transparency of the air-bubble) than any other that was examined. Varying amounts are present in succeeding figures. Mean centrifugal field, 4,700 gravity.

FIG 2 The sedimentation diagram of an active bacteriophage preparation after standing for 3 days (in the cold) at pH 10. Note the faint boundaries due to the heavy component and the large amount of unsedimentable material. Mean field = 5,400 gravity.

FIG 3 The sedimentation diagram of an active preparation that contained an unusually large amount of unsedimentable material. The boundaries respond to  $s_{20}^0 = ca. 160 \times 10^{-13}$ . A minute leak in the cell is indicated by the gradually widening air-bubble. Mean field = 5,500 gravity.

FIG 4 The diagram of an exceptionally active preparation in a dilute sulfate solution of density 1.12. Note the diffuseness of the boundary of the heavy component. Mean field = 25,000 gravity.

FIG 5 The diagram of the same preparation as that of Fig 4 in a more concentrated sulfate solution of density 1.04. Note the increased sharpness of the boundary. Mean field = 11,000 gravity.

## PLATE 3

FIG 6 The diagram of a preparation inactivated by heating for 10 minutes at 60 C. The extraordinarily sharp boundaries of the sedimenting gel are marked (d). Mean field = 146,000 gravity

FIG 7 The diagram of the inactive preparation of Fig 6 after remixing from previous centrifugation. The boundary is less sharp than before and it settles faster. Mean field = 144,000 gravity

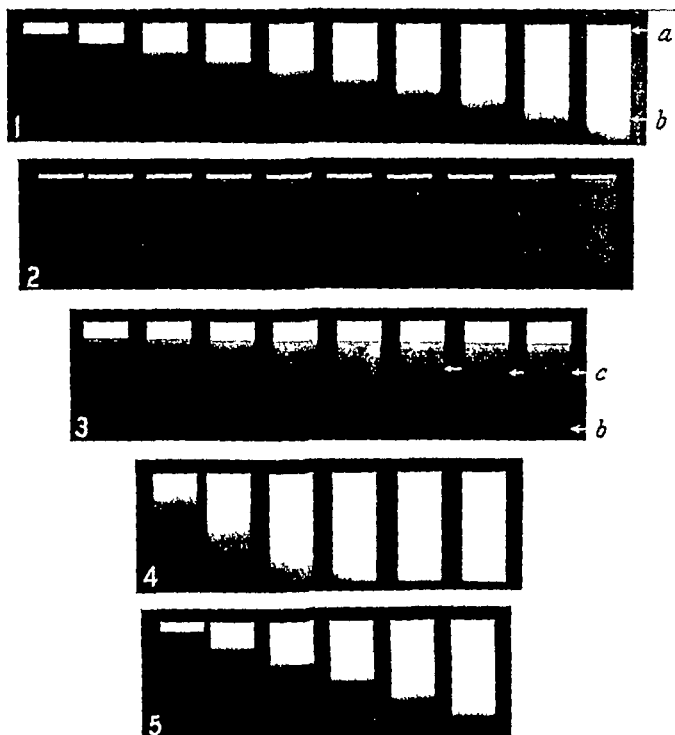
FIG 8 The diagram of another heat inactivated preparation sedimenting in an ammonium sulfate solution of density 1.10. The diffuse boundaries (e) correspond to  $s_0 = ca\ 3 \times 10^{-13}$ . Diagrams indistinguishable from this except for increased rates of sedimentation have been obtained from heat inactivated solutions containing little ammonium sulfate. Mean field = 125,000 gravity

FIG 9 The diagram of the preparation of Fig 8 after remixing. The new boundary (d') sediments nearly twice as fast as before and disappears after the next few pictures. Mean field = 125,000 gravity

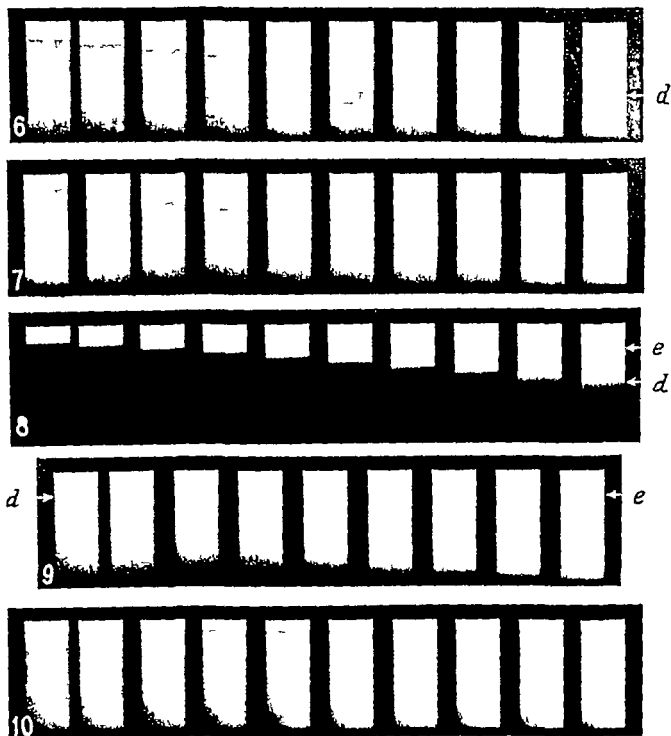
FIG 10 The diagram of a preparation inactivated by chymo trypsin. Note the comparative diffuseness of the faint heavy boundary and the large amount of unsedimentable material. Mean field = 5,400 gravity













## DARK ADAPTATION IN DINEUTES\*

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Studies of dark adaptation have been made on amoeba (Folger, 1925), *Volvox* (Mast, 1927), some molluscs (Hess, 1910, Hecht, 1918, 1919, 1923, 1927, Crozier and Wolf, 1928), an ascidian (Hecht, 1918, 1927), *Amphioxus* (Hess, 1910), the frog tadpole (Obreshkove, 1921, Hecht, 1927), the chick (Honigsmann, 1921), and man (for references see Hecht, 1921, 1927, and Hecht, Haig, and Wald, 1935), but until recently (Hartline, 1929, Wolf and Zerrahn Wolf, 1935) little information was available describing the course of dark adaptation in arthropods in any but the most general terms (Hess, 1910, Dolley, 1929, von Buddenbrock and Schulz, 1933)

This paper reports a study of dark adaptation in the aquatic beetle, *Dineutes assimilis* commonly called apple bug, coffee bug, or whirligig beetle. It was found (Clark, 1931, 1933) to be an excellent form for study. It reacts well to light, is hardy and easily kept in the laboratory, and can, if necessary, be used in repeated experiments.

### *Apparatus and Procedure*

Starting with the observation that a specimen of *Dineutes* in the dark tends to move around the walls of a container, an apparatus was devised by which all the ommatidia could be about equally light adapted and in which the insect could be oriented in the same relation to the stimulating light for each experimental reading.

The apparatus (Fig. 1) consisted of a turn table composed of a ring moving on ball bearings and supporting a sheet of bakelite with a square opening in the center. A piece of ground glass set flush with the bakelite covered the opening. An animal compartment was formed by cementing clear glass walls to the ground glass. The turn table was mounted to allow light to be reflected up through the ground glass after being transmitted through a neutral tint filter or through a red filter. The former was the adapting light, and the latter was used to view

\* Investigation pursued during tenure of National Research Fellowship

the animals while taking readings, as they are insensitive to the deep red of the spectrum

Another lamp was mounted to give a horizontal beam, the intensity of which could be controlled by a neutral tint wedge and filters. This was the test light

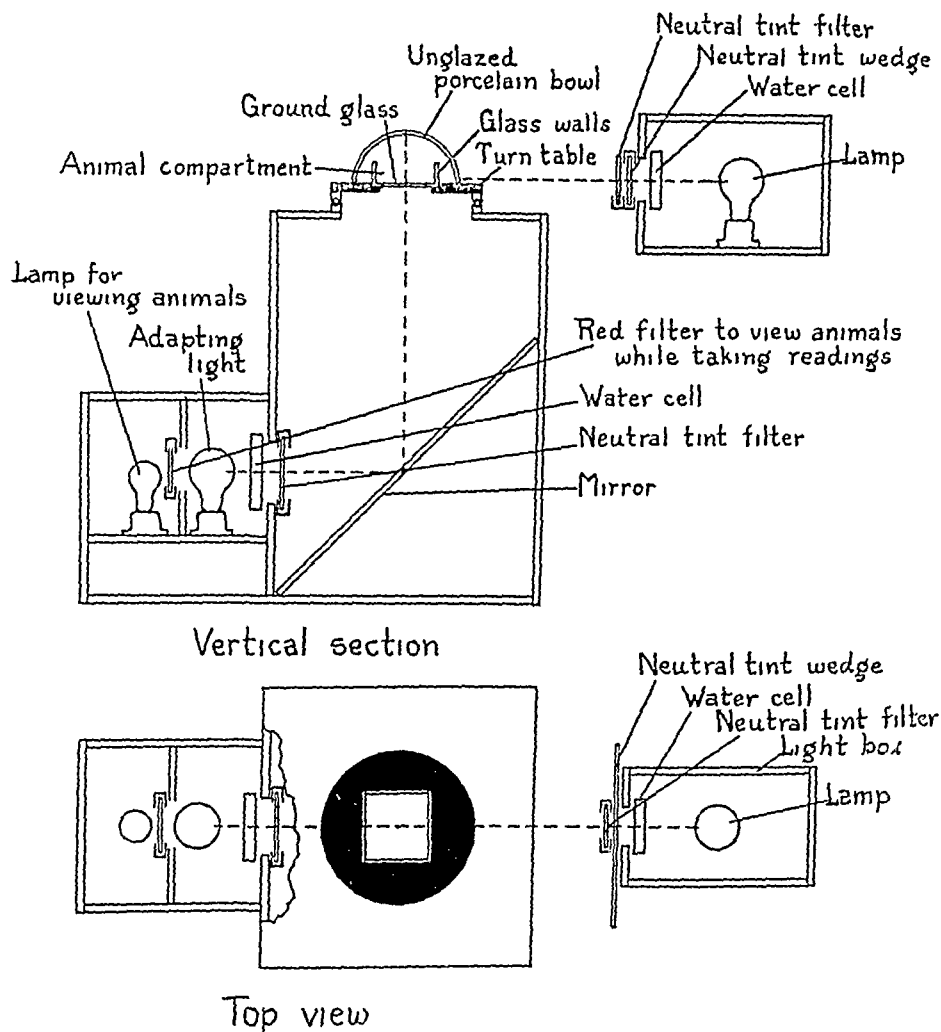


FIG 1 Diagram of apparatus used to test dark adaptation of *Dineutes*

The experimental procedure was as follows. An insect was placed in the animal compartment and a white unglazed porcelain bowl inverted over the chamber. The adapting light was turned on and the insect was adapted to light passing through the ground glass floor and reflected from the white walls of the bowl. This scattered the light so the intensity was about equal from all directions.

After light adaptation, the adapting light was turned off, the bowl removed, and the animal left in the dark for a known time. Then the minimum intensity to which the insect would react was determined. The red light was turned on to view the animal. The insect usually moved around the walls. If it was moving along the wall away from the test lamp and at right angles to the beam, the test lamp was turned on and the intensity was slowly increased until the insect turned toward the light. If the insect reached the side wall of the compartment and started along it parallel to the beam before it reacted, the turn table was moved one quarter turn to cause the insect to move across the beam again. At some intensity the insect will turn and move toward the light. The minimum intensity calling forth this reaction is called the threshold or threshold intensity and is given as  $\log I + 10$ .

When the threshold was determined the time was noted and the test lamp turned off. The insect was left in the dark for a further timed period and then tested again. Thus the threshold was determined at the end of a number of timed periods in the dark during the course of dark adaptation. This was repeated with each of several insects.

#### RESULTS

Experiments were performed using light adaptation of 6.5, 91.6, and 6100 foot candles, and curves of dark adaptation secured for each. The results are presented in Table I. Each determination is a single reading from one individual.

The data for each insect behave in the same regular manner. As the intensity of the adapting light increases, the early threshold values also increase. Though the course of dark adaptation is about the same for each adaptation level, the final thresholds may vary. To avoid averaging, the data of each set of readings from each insect are moved as a whole up or down the  $\log I$  axis to bring them all to approximately the same threshold. This introduces no change in the curves of dark adaptation but does cause a change in the units in which intensity is measured. The shift is made by multiplying the threshold intensity by a constant  $a$ . This constant is probably related to the condition of the nervous system as well as to the transmission of the eye pigments, since it may vary from day to day in the same individual (see insect V, experiments 1 and 2 at light adaptation of 91.6 foot candles). The value of  $\log a$  for each set of data is given in Table II.

By plotting the  $\log$  threshold  $+ \log a$  values against time in the dark and by using the appropriate time intervals on the abscissa, all data

### Light adaptation to 6100 foot-candles



TABLE II

Values of  $\log a$  to Bring Data of All Animals to Approximately Same Final Threshold

Insect	$\log a$	Insect	$\log a$	Insect	$\log a$
III-1	-0.20	IV-6	0.15	XIII-2	0.00
IV-1	0.12	V-1	-0.73	XIV-1	0.00
IV-2	0.15	V-2	-0.24	XV-1	-0.35
IV-4	0.00	VI-1	-0.23	XVI-1	0.22
IV-5	0.00	XIII-1	0.20	XVII-1	-0.05

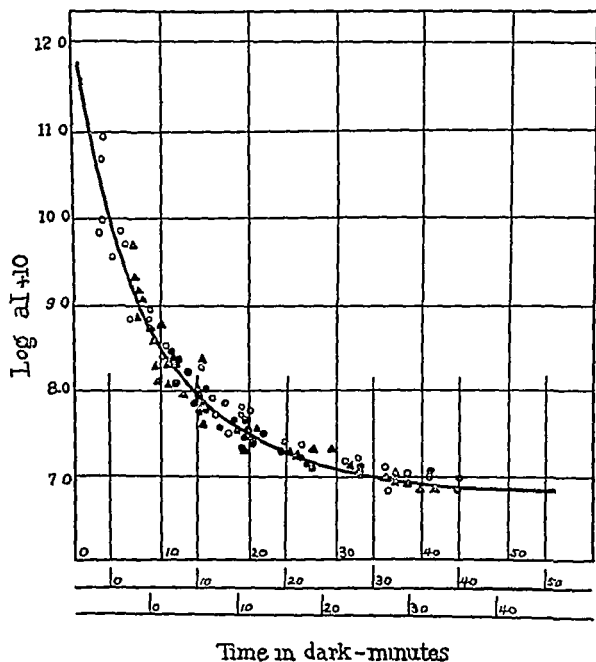


FIG. 2. Curve of dark adaptation of *Drosophila*. Open circles, triangles, and closed circles, individual observations after light adaptation to 6100 foot candles, 91.6 foot candles, and 6.5 foot candles respectively.

can be described by a single curve (Fig. 2) This indicates that the course of dark adaptation follows parts of the same curve irrespective of the level of light adaptation The intensity of the adapting light determines the level at which dark adaptation will begin

It is obvious that it is extremely difficult to secure experimental values for the so called instantaneous threshold, the threshold at zero time in the dark But they may be readily secured from the curve of Fig 2 When the instantaneous  $\log aI$  is plotted against

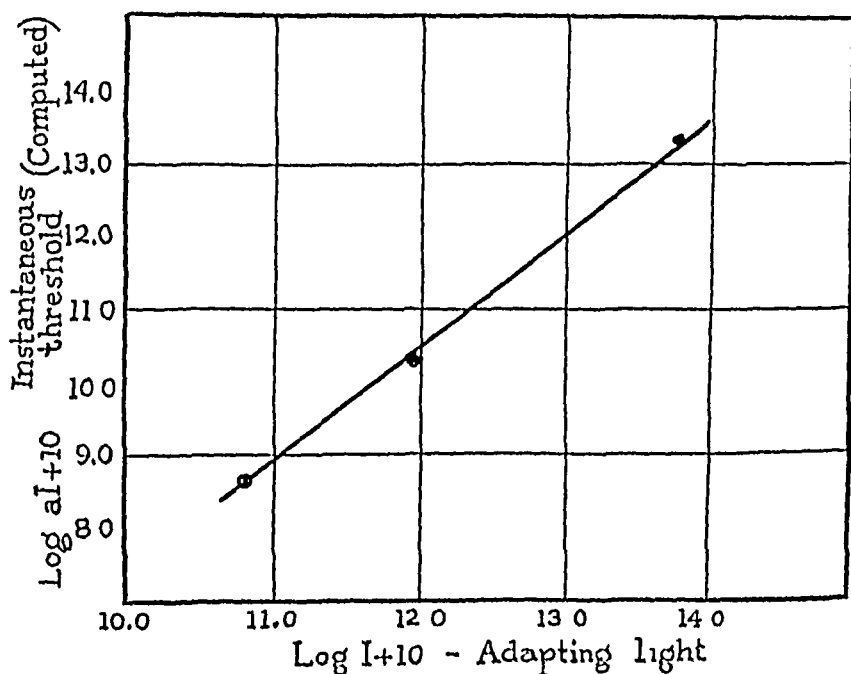


FIG 3 Relation between log of instantaneous threshold and log of adapting intensity

the log of the adapting light, the result is as shown in Fig 3 Although the relation seems linear, it is more likely that the range of intensities covered falls in the central part of a sigmoid curve similar to that found for certain other organisms

### *Theory*

On analysis the data were found to be described by the equation for human dark adaptation derived by Hecht (1929) on the assumption that the sensitive material is formed from two of its photoprod-

ucts, and that the concentration of sensitive material is inversely proportional to the logarithm of the threshold intensity at the moment. The equation is

$$k_2 t = \frac{\log aI_t \times \log aI_\infty}{\log aI_t - \log aI_\infty} + C$$

where  $k_2$  may be considered similar to a velocity constant,  $t$  is the time in the dark,  $\log I_t$  is the log of the threshold intensity at time  $t$ ,  $\log I_\infty$  is log of threshold intensity at complete dark adaptation, and  $a$  is the constant described above.

Computations of the individual points yielded average values of  $k_2 = 1.32, 1.48$ , and  $1.28$  for the three series. These values are most probably not significantly different, a conclusion shown by the drawing of a common curve through all data. The average  $k_2$  for all points was  $1.37$  and was used in securing the curve of Fig. 1.

#### CONCLUSION

Theory apart, it is obvious that (a) all the data fall on one curve and merely represent different starting levels on the same curve of dark adaptation, and (b) the relation between the log instantaneous threshold and log adapting light is linear over the range covered.

#### SUMMARY

The level of dark adaptation of the whirligig beetle can be measured in terms of the threshold intensity calling forth a response.

The course of dark adaptation was determined at levels of light adaptation of 6.5, 91.6, and 6100 foot candles. All data can be fitted by the same curve. This indicates that dark adaptation follows parts of the same course irrespective of the level of light adaptation. The intensity of the adapting light determines the level at which dark adaptation will begin.

The relation between  $\log aI_0$  (instantaneous threshold) and  $\log$  of adapting light intensity is linear over the range studied.

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# THE CONCENTRATION EFFECT WITH VALONIA POTENTIAL DIFFERENCES WITH DILUTED POTASSIUM RICH SEA WATERS

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This report is the fifth in a series<sup>1</sup> from this laboratory dealing with the concentration effect in large plant cells. The present paper is concerned with the changes in  $P.D.$  across the protoplasm of cells of *Valonia macrophysa* observed<sup>2</sup> when these cells were exposed to various dilutions of certain artificial solutions resembling sea water, but in which the  $NaCl$  of sea water was more or less completely replaced by  $KCl$ . Electrical effects produced by  $KCl$  are of especial interest in view of the remarkable degree to which this salt is accumulated in *Valonia* sap. These new experiments serve as a further test of the interpretation of bioelectric potentials which has been developed in earlier papers, and which is now summarized in the following paragraphs.

In the measurements of  $P.D.$  with *Valonia*, the cell is impaled on a fine glass capillary filled with artificial sap, through which electrical connection is established with the interior of the cell. The  $P.D.$  across the protoplasm is thus measured directly between the vacuolar sap and an external solution bathing the entire outer surface of the cell. The advantages of this procedure have been discussed in earlier papers.<sup>1b, 3</sup>

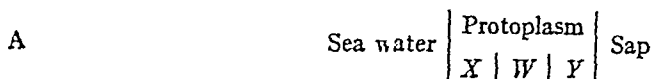
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<sup>1</sup> Earlier papers (a) Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 761 (b) Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 445 (c) Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715, (d) Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, 20, 13.

<sup>2</sup> The experiments reported in this paper were carried out at Bermuda in 1931. Apparatus and methods for supporting the impaled cells applying the solutions and measuring the  $P.D.$  have been described in earlier publications.<sup>3</sup>

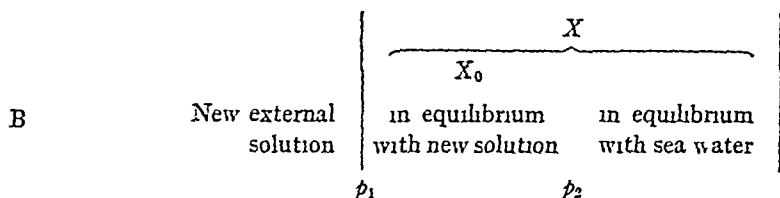
<sup>3</sup> Damon, E. B., *J. Gen. Physiol.*, (a) 1929-30, 13, 207 (b) 1931-32, 16, 525 (c) 1932-33, 16, 375.

Experiments indicate that the protoplasm consists<sup>4</sup> of an aqueous layer ( $W$ ) bounded by two dissimilar non-aqueous surface layers ( $X$  and  $Y$ ). The system which is measured when the cell is immersed in natural sea water may accordingly be represented as follows



It is assumed that the concentrations of electrolytes are uniform throughout the  $X$  layer, in distribution equilibrium with sea water

When some other external solution is substituted for sea water, we may make the usual assumption that a thin film at the outer surface of  $X$  comes at once into distribution equilibrium with the external solution, this film will be called  $X_0$ . If this assumption is correct, we shall have at the start in  $X$



while the rest of the protoplasm remains for the time being unchanged. We may accordingly attribute the initial change in  $P D$  to the new diffusion potential at  $p_2$ , plus any change in the phase boundary potential at  $p_1$  (provided the measurement is made before diffusion has had time to penetrate through the  $X$  layer into  $W$ ). Since we shall consider only this initial change, the potentials at the other layers of the protoplasm need not enter into our calculations.

According to the usual theories of phase boundary potentials, the  $P D$  at  $p_1$  is given by the expression<sup>5</sup>

$$P D = \frac{RT}{2F} \ln \frac{A_K C_K + A_{Na} C_{Na} +}{A_{Cl} C_{Cl} +} \quad (1)$$

where  $C_K$ ,  $C_{Na}$ , and  $C_{Cl}$  represent the concentrations of the  $K^+$ ,  $Na^+$ , and  $Cl^-$  ions in the external solution, and  $A_K$ ,  $A_{Na}$ , and  $A_{Cl}$  the "true" ionic partition coefficients. ( $Ca^{++}$ ,  $Mg^{++}$ , and  $SO_4^{--}$  ions, also present in the solutions used in these experiments, need not be included in Equation 1, since it has been shown<sup>1b</sup> that the  $P D$  observed with diluted sea water is not affected by relatively large changes in the concentrations of these ions. It may therefore be assumed that the partition coefficients for these ions are negligibly small.) In the present

<sup>4</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, 11, 83, *Biol. Rev.*, 1931, 6, 369, *Ergeb. Physiol.*, 1933, 35, 1013

<sup>5</sup> Michaels, L., and Fujita, A., *Z. phys. Chem.*, 1924, 110, 266, Horovitz, K., *Z. phys. Chem.*, 1925, 115, 424

discussion, we shall adopt the assumption (used also in an earlier paper<sup>1b</sup>) that the true ionic partition coefficients are constant independent of changes in concentration. It then follows from equation (1) that the phase boundary potential at  $p_1$  will not be affected by changes in the dilution of the external solution and hence that changes in the P.D. resulting from dilution alone may be ascribed to the diffusion potential at  $p_2$ .

The data obtained from measurements of concentration effect may accordingly be used as a basis for calculating the relative mobilities of ions in the  $\gamma$  layer. This calculation was simplified by the discovery<sup>1b</sup> that the concentration effect observed with natural sea water is practically the concentration effect for NaCl alone, the part played by the other salts of sea water being relatively small. (The presence of these salts is nevertheless of great importance for preventing injury to the protoplasm.) Interpretation of the concentration effect with natural sea water as a diffusion potential at  $p_2$  involving only the  $\text{Na}^+$  and  $\text{Cl}^-$  ions has led to the conclusion<sup>1b</sup> that the mobility of  $\text{Cl}^-$  in the  $\gamma$  layer is about five times as great as that of  $\text{Na}^+$ .

The relative mobility of  $\text{K}^+$  in the  $X$  layer has been calculated from the changes in P.D. observed when *Valonia* cells were transferred from natural sea water to artificial sea waters in which all or part of the NaCl was replaced by KCl, it was found<sup>3c</sup> that the mobility of  $\text{K}^+$  is about twenty times as great as that of  $\text{Cl}^-$ . In this calculation, the additional assumption was made that the true ionic partition coefficients for  $\text{K}^+$  and  $\text{Na}^+$  are equal. Obviously, other assumptions as to the magnitude of the partition coefficients will lead to different values for the mobility of  $\text{K}^+$ . It has been demonstrated<sup>3c</sup>, however, that no values which may be assigned to  $A_K$  and  $A_{Na}$  in equation (1) will account for the observed P.D. on the basis of phase boundary potentials alone. In any case a large proportion of the observed change must be assigned to the diffusion potential at  $p_2$ . It is therefore highly probable, whatever the partition coefficients may be, that the mobility of  $\text{K}^+$  in the  $X$  layer is actually greater than that of  $\text{Cl}^-$ .

From the apparent relative mobilities of  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  in the outer surface layer of *Valonia* protoplasm ( $K > Cl > Na$ ), it is to be expected that with artificial sea waters containing high concentrations of KCl the sign of the concentration effect will be opposite to that with natural sea water. That is, whereas with natural sea water we find that dilution increases the inwardly directed P.D.<sup>6</sup> across the protoplasm, with KCl rich sea waters it is to be expected that dilution will decrease the inwardly directed P.D. The new experiments

<sup>6</sup> With natural sea water and with the modified and diluted sea waters used in the experiments reported in this paper, the P.D. across the protoplasm of *Valonia* is directed inward, in the sense that positive current tends to flow from the external solution through the protoplasm into the vacuolar sap.

show that this is actually the case with small dilutions of the KCl-rich sea waters. With higher dilutions, however, more complicated phenomena are encountered which make it necessary to modify some of the simple assumptions employed in the foregoing discussion.

The measurement of the concentration effect with KCl-rich sea waters is complicated by the fact that the  $P/D$  with these solutions does not remain constant, but undergoes more or less characteristic changes<sup>3c</sup> which have been ascribed to the penetration of  $K^+$  into the  $W$  and  $V$  layers of the protoplasm. These changes begin very soon after the KCl-rich solution has come in contact with the protoplasm. Consequently, when a cell is treated first with a KCl-rich sea water, and then with a dilution of this sea water, it is very difficult to distinguish between changes in  $P/D$  actually produced by dilution and such fluctuations as would have occurred if the solution had remained unchanged.

A study of  $P/D$ -time curves has shown, however, that when natural sea water is replaced by undiluted KCl-rich sea water the initial rise in  $P/D$  is reproducible and can be calculated from the concentrations of  $K^+$  in the sea waters (although subsequent changes in the  $P/D$  may be complex). It appears, therefore, that the best method available for studying the effect of diluting these solutions is to compare the initial changes in  $P/D$  across the protoplasm of impaled cells when different dilutions of KCl-rich sea water are substituted for natural sea water.

The  $P/D$ -time curves in Fig. 1 illustrate the kind of data which can be obtained by this procedure. (The differences in the forms of these curves will be discussed later.) The solutions used in these measurements were dilutions of a KCl-rich sea water<sup>7</sup> (hereafter called KCl-

<sup>7</sup> The modified sea waters used in these experiments had the following composition, based on a formula for artificial sea water recommended by McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. No. 251*, 1917.

K + Na	0.500 molar	Cl	0.570 molar
Ca	0.011 "	Br	0.001 "
Mg	0.054 "	SO <sub>4</sub>	0.028 "
		HCO <sub>3</sub>	0.003 "

Dilutions of these modified sea waters were made with a solution of glycerol, 8.7 per cent by weight, in distilled water. The freezing point of this solution is approximately the same as that of Bermuda sea water.



sea water) in which the concentration of KCl was 0.500 molar, all the NaCl of ordinary sea water being replaced by KCl. The data obtained from these and other similar experiments are collected in

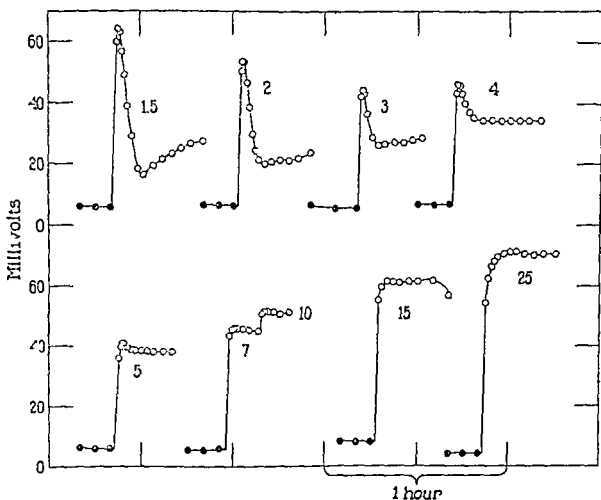


FIG. 1. Typical P.D. time curves showing changes in the P.D. across *Valonia* protoplasm when different dilutions of KCl sea water (open circles) were substituted for natural sea water (shaded circles) as the external solution. The number at the right of each curve indicates the dilution,  $\pm e$ , the number of liters of dilute solution containing one liter of KCl sea water.

The five measurements using dilutions up to fivefold were made on the same *Valonia* cell, the four measurements with higher dilutions were made on a second cell.

The P.D. is in all cases directed inward;  $\pm e$  positive current tends to flow from the external solution through the protoplasm into the vacuolar sap.

Fig. 2, curve A, which shows the relation between the dilution of KCl sea water and the initial rise in P.D. when diluted KCl sea water replaces natural sea water.

With dilutions less than fivefold, the initial rise in P.D. was found in

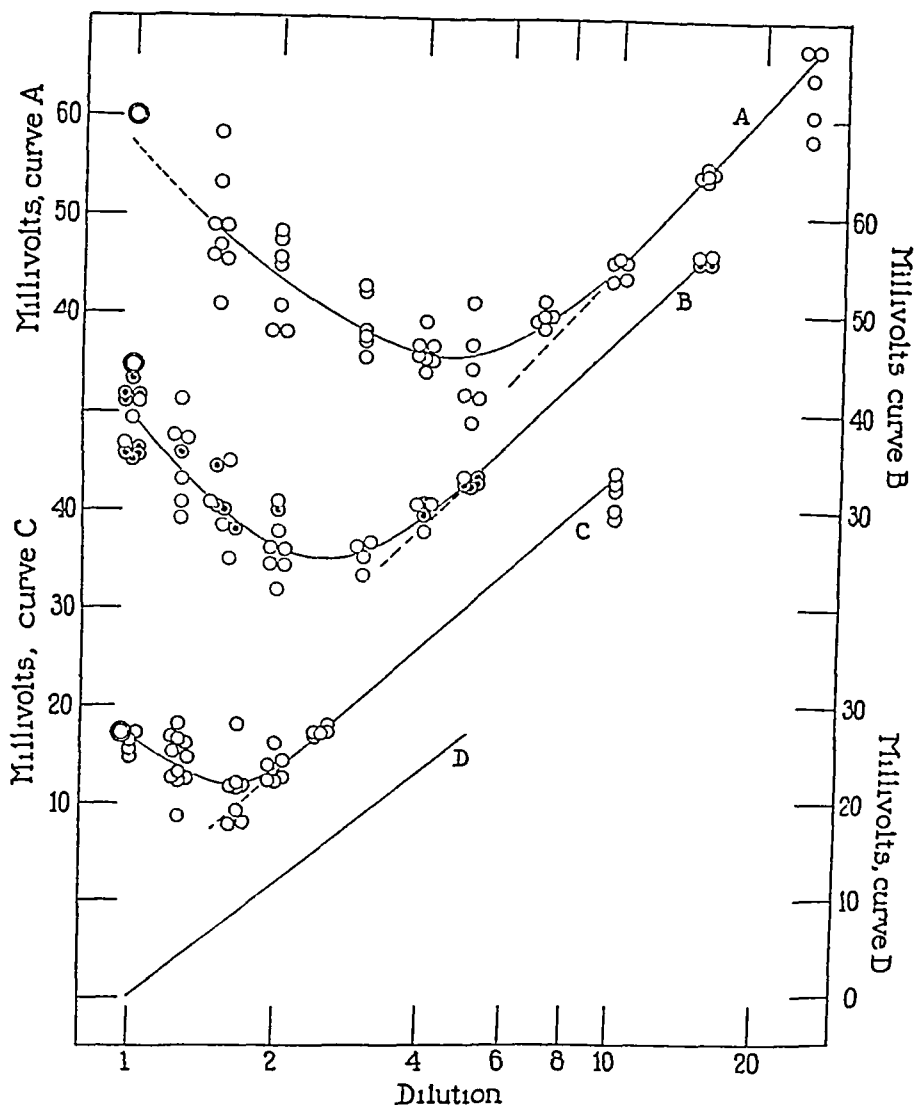


FIG 2 Curves showing the concentration effect with dilutions of KCl-rich sea waters in *Valonia*. P.D.'s plotted as ordinates are differences between the P.D. in ordinary sea water and the first maxima in the P.D.-time curves when natural sea water was replaced by diluted KCl-rich sea water. The dilutions are plotted on a logarithmic scale as abscissae.

Data for each curve were obtained from measurements on 15 or 16 individual cells. Each small circle represents a single measurement, in cases where a measurement was repeated on the same cell, only the highest value is reported. The large double circles show the average values of P.D. with the undiluted sea waters as reported in an earlier paper<sup>3c</sup>. Each group of points represents measure-

general<sup>8</sup> to decrease with increasing dilution. This is in agreement with previous results<sup>3c</sup> showing that the mobility of  $K^+$  in the outer layer of the protoplasm is greater than that of  $Cl^-$ . With dilutions greater than fivefold, however, the behavior was altogether different: the  $P/D$  time curves were of an obviously different form, the initial rise in  $P/D$  was larger than with the fivefold dilution, and the  $P/D$  increased regularly as the dilution was increased (Fig 1, Fig 2A). In other words, with dilutions greater than 5, the sign of the concentration effect with KCl sea water is reversed, becoming the same as with natural sea water.

The precise dilution at which the change in type of  $P/D$ -time curve occurs was found to vary slightly from cell to cell. Thus, in several cases, the curve with the fivefold dilution of KCl sea water had the shape characteristic of higher dilutions, in one case, the curve with the sevenfold dilution resembled the curves with dilutions less than 5. In such cases, the values of the initial rise in  $P/D$  were in fair agreement with the values observed with time curves of the more usual type. It is perhaps significant, however, that the lowest values of  $P/D$  with the fivefold dilution were obtained from curves of the type commonly found with higher dilutions.

Similar phenomena were observed with KCl rich sea waters containing smaller proportions of extra KCl. Curves *B* and *C* of Fig 2 show the results of measurements like those reported in Fig 1 and

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ments with the same solution, but in order to show all observed values a number of pairs of points have been displaced symmetrically to right and left of their true abscissae.

Curves have been fitted approximately to the average values of  $P/D$  in so doing closely grouped points have been given more weight than scattered points and among scattered points high values have been given more weight than low ones.

*A* shows the concentration effect with KCl sea water containing 0.500 mole of KCl in a liter. *B*, with the KCl rich sea water containing 0.200 mole of KCl. *C* with the KCl rich sea water containing 0.050 mole of KCl. *D* shows, for comparison, the concentration effect for natural sea water ( $KCl = 0.012$ ) as reported in an earlier paper.<sup>1b</sup>

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<sup>8</sup> In Fig 1, however, the value of the initial rise with the threefold dilution falls somewhat out of line with the other values measured on the same cell.

Fig 2A, but using dilutions of two modified sea waters in which the concentrations of potassium were respectively 0.200 and 0.050 molar. With these solutions, as with KCl-sea water, the change in the sign of the concentration effect was accompanied by a change in the form of the  $P D$ -time curves. It will be seen that the smaller the concentration of potassium in the undiluted sea water, the lower is the dilution at which the change in the sign of the concentration effect occurs. This relationship will be discussed further on.

In the measurements with diluted KCl-sea water, the pH of the solutions was adjusted to the same value as that of ordinary sea water, as indicated by the color of cresol red. This procedure was modified later, after a study of the effect of varying the reaction of undiluted natural and KCl-rich sea waters<sup>3c</sup>. It was found that changing the pH between 5 and 10 does not affect either the  $P D$  with natural sea water or the initial rise in  $P D$  when natural sea water is replaced by KCl-rich sea water. The changes in  $P D$  with KCl-rich solutions, however, are considerably slower when the reaction is acid than when it is alkaline. In the measurements with the other two KCl-rich sea waters, therefore, the pH of the solutions was lowered to between 5 and 6, in order to broaden the first peak of the  $P D$ -time curve, and thus increase the probability of observing the full value of the change in  $P D$ .

The measurements were carried out at room temperature. In the experiments with KCl-sea water (March, 1931) this varied between 16° and 22°, average, 18°. In the majority of the measurements with the other two KCl-rich sea waters (May) the temperature was  $23^{\circ} \pm 2^{\circ}$ , in a few additional measurements with dilutions less than the critical dilution (July–August) the temperature was  $28^{\circ} \pm 1^{\circ}$ . Fortunately, the temperature coefficient of the  $P D$  appears to be small.

With the *Valonia* cells used in these measurements, the  $P D$ 's with the undiluted and slightly diluted KCl-rich sea waters appear to be rather low, compared with the values previously reported<sup>3c</sup> for the undiluted solutions. This may be seen in Fig 2, where averages of earlier values are plotted as double circles. A particularly large number of cells exhibiting a small potassium effect was encountered in the measurements with dilutions of the sea water containing 0.200 molar KCl. In this series, therefore, it was decided to include only cells which when treated with the undiluted solution showed an initial rise in  $P D$  of at least 35 mv.

In connection with these lower  $P D$ 's, it may be significant that at this time (May, 1931) it proved especially difficult to keep the impaled cells free from a gelatinous coating of marine bacteria. This coating greatly retards diffusion, and in time leads to injury and death of the cell. Even before injury has actually occurred, the presence of a film of bacteria may greatly increase the time required for leaching out the cell wall and bringing the protoplasm in contact with a new solution of the desired composition.

In measurements with the more dilute solutions, where the  $P.D.$  remains constant after it reaches a definite level, the result may be merely an increase in the time required for the  $P.D.$  to reach its final value. With undiluted or moderately diluted KCl rich sea water, however, where the  $P.D.$  passes through a maximum, it becomes essential to apply the solution as rapidly as possible in order to observe the full value of the initial rise in  $P.D.$ , here the observed value may be greatly diminished by the presence of bacteria.

The removal of bacteria from impaled cells by such methods as washing with a stream of sea water is not feasible because of the danger of injury to the seal between the protoplasm and the glass capillary. Accordingly, no measurements were accepted with any cell which had become visibly coated with bacteria.

A key to the explanation of the reversal of the sign of the concentration effect with KCl rich sea waters may be found in a comparison of the shapes of the  $P.D.$  time curves with different dilutions of KCl sea water as shown in Fig. 1.

With small dilutions, the  $P.D.$  time curves have approximately the same form as with undiluted KCl sea water. After the initial rise, the  $P.D.$  falls rapidly to a minimum, then rises again more slowly to a second maximum. This characteristic fluctuation of the  $P.D.$  (found also with *Valonia* sap and with pure 0.6 molar KCl) has been interpreted<sup>3</sup> as due to the penetration of KCl into the aqueous middle layer of the protoplasm. In accordance with this interpretation, we may conclude that with dilutions less than 5 (Fig. 1) the initial increase in  $P.D.$  results mainly from the high relative mobility of  $K^+$  which is diffusing inward through the non aqueous outer surface layer of the protoplasm.

As the dilution is increased, the fluctuations in  $P.D.$  become progressively less, which suggests that the amount of KCl entering the protoplasm is becoming smaller. Finally, with dilutions greater than 5, the curves become altogether different from the curves with undiluted KCl rich sea waters. The fluctuations attributed to the entrance of KCl are no longer perceptible.

With these higher dilutions of KCl sea water, where the concentration effect has the same sign as with natural sea water, the  $P.D.$  time curves have the same shape as the curves observed with dilutions of natural sea water<sup>1b, 9</sup> the  $P.D.$  rises rapidly to a definite value at which it remains approximately constant for some time (until secondary changes in the cell appear as a result of too prolonged exposure to

<sup>9</sup> A typical  $P.D.$  time curve with diluted natural sea water is shown in Fig. 3.

dilute solutions) This change in the form of the  $P D$ -time curves suggests that with the higher dilutions of KCl-rich sea waters potassium no longer plays an important part in the  $P D$ , and that the concentration effect with these solutions is to be interpreted in the same way as the concentration effect with dilutions of natural sea water

If the concentration effect with the higher dilutions of KCl-rich sea waters is to be explained as similar to that with natural sea water, it becomes necessary to examine more closely the assumptions which are involved in the interpretation of the concentration effect with natural sea water

In this discussion, only the  $K^+$ ,  $Na^+$ , and  $Cl^-$  ions need be considered It has been shown<sup>1b</sup> that changes in the concentrations of the other principal ions of sea water have little or no direct influence on the  $P D$  with diluted sea water

This was demonstrated by comparing the  $P D$ 's across the protoplasm of a *Valonia* cell when the external solutions were (1) a threefold dilution of sea water (in which the concentrations of all the salts were reduced by the same factor) and (2) an artificial solution in which the stoichiometric concentrations of Na and Cl were the same as in the diluted sea water while the concentrations of the other principal ions were approximately the same as in undiluted sea water It was found that the  $P D$ 's with these two solutions were almost identical, the value with the second solution (23.5 to 24.6 mv) being slightly larger than that with the diluted sea water (23.0 to 23.8 mv) That is, the effect of a threefold reduction in the concentrations of Na and Cl alone was actually somewhat greater than the effect of a similar reduction in the concentrations of all the ions The reason for this discrepancy is that, although the concentrations of Na and Cl were the same in the two solutions, the activity of NaCl was somewhat greater in the diluted sea water (solution 1), corresponding to the lower ionic strength of this solution A quantitative test of this explanation will be presented later (page 396)

Since the other principal ions of sea water ( $Mg^{++}$ ,  $Ca^{++}$ ,  $SO_4^{--}$ , etc.) appear to play a negligible part in the  $P D$ , we may suppose that in the outer or  $X$  layer of the protoplasm the concentrations of these ions, or their mobilities, or both, are very small, so that the products of concentration multiplied by mobility may be neglected in comparison with the corresponding products for  $Cl^-$ ,  $Na^+$ , and  $K^+$

It has been inferred from the shape of the  $P D$ -time curves that  $K^+$  likewise plays no important part in the concentration effect with

natural sea water or with the higher dilutions of KCl rich sea waters. This assumption is supported by experiments which show that small changes in the concentration of  $K^+$  in diluted sea water have little or no effect on the p.D. across *Valonia* protoplasm. Such measurements are presented in Fig. 3, where the p.D. with a threefold dilution of natural sea water is compared with the p.D. with an equal dilution of

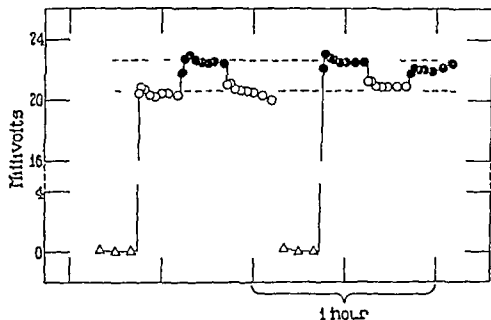


FIG. 3 P.D. time curves, from measurements on two different cells, illustrating the effect of moderate changes in the potassium content of sea water diluted with an isotonic solution of glycerol. Triangles represent P.D.'s in undiluted sea water, arbitrarily taken as zero, open circles P.D.'s in a threefold dilution of natural sea water, shaded circles, P.D.'s in a threefold dilution of a KCl rich sea water. Molar concentrations in the dilute solutions were

1/4 natural sea water	K, 0 004	Na, 0 163	Cl, 0 191
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$\frac{1}{2}$ KCl in sea water	K, 0 033	Na, 0 133	Cl, 0 191
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Horizontal broken lines indicate the calculated difference of 2.0 mv (page 396). To reduce the size of the figure the scale of ordinates is broken between 4 and 16 mv. For the meaning of "dilution" see Fig. 1.

a KCl rich sea water containing 0.100 mole of KCl in a liter (The concentrations of  $K^+$  in the diluted solutions were respectively 0.004 and 0.033 molar) It will be seen that the p.d.'s with these two dilute solutions were not more than 2 mv apart Not only is this effect a small one, but as we shall see presently (page 396) it can be accounted for satisfactorily as resulting from the unequal concentrations of Na in the two solutions Thus (within certain limits, to be discussed later)

it appears that when K is substituted for Na in diluted sea water, the P D is affected only by the decrease in the concentration of Na, and not at all by the increase in the concentration of K

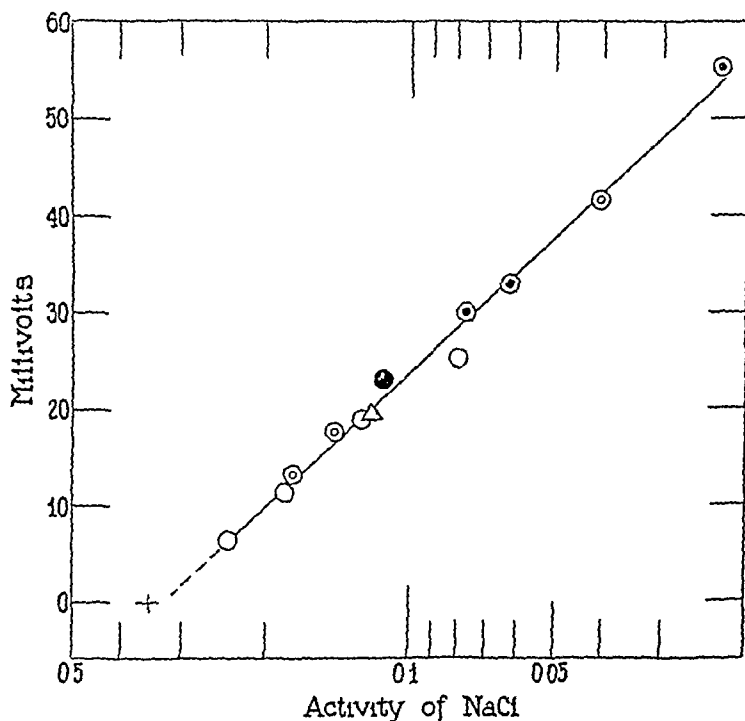


FIG 4 Curve showing the relation between the P D and the activity of NaCl in dilutions of natural sea water and in certain higher dilutions of KCl-rich sea waters. The mean ionic activities of NaCl in the external solutions are plotted as abscissae on a logarithmic scale, decreasing from left to right. Ordinates show the initial rise in P D when natural sea water was replaced by the solution in question. The solutions are (1) dilutions of natural sea water, as reported in an earlier paper<sup>1b</sup>—open circles, (2) the three highest dilutions of the sea water with 0.050 molar KCl, from Fig 2C—double circles, open centers, (3) the threefold dilution of sea water with 0.100 molar KCl, from Fig 3—shaded circle, (4) the three highest dilutions of the sea water with 0.200 molar KCl, from Fig 2B—double circles, shaded centers, (5) the  $\text{Na}^+_{1/3}, \text{Cl}^-_{1/3}$  sea water described in an earlier paper<sup>1b</sup>—triangle. The method of least squares was used to determine the best straight line passing through these points. The P D with ordinary sea water, taken as zero, is represented by a cross, this point does not lie on the curve (see page 405).

By elimination, therefore, it is found that the concentration effect with higher dilutions of KCl-rich sea waters, as well as with natural sea water, is practically the concentration effect for NaCl alone. This



conclusion may be tested, in the case of sea waters containing appreciable concentrations of Na, by plotting the observed  $P D$  as a function of the activity of NaCl in the external solution. Fig 4 shows the result of such a test, using the data for  $P D$  with diluted natural sea water<sup>13</sup> and with those higher dilutions of KCl rich sea waters where the  $P D$  time curves were of the same type as with diluted natural sea water. The values of the mean activity<sup>10</sup> of NaCl in these solutions, plotted as abscissae on a logarithmic scale, varied by tenfold (from 0.24 to 0.023).

It is evident that the concentration effect over this entire range is governed by the activity of NaCl in the external solution. Since the concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  were varied independently in these sea waters, the test shows that both ions are directly concerned in the concentration effect. The  $\text{K}^+$  ion, on the contrary, is found to have no perceptible influence on the  $P D$  with these solutions, although the mean activity of KCl varied by fivefold (from 0.012 to 0.060).

By analogy with familiar equations for  $P D$  the empirical equation represented by the straight line of Fig 4 may be written in the form

$$\begin{aligned} P D_2 - P D_1 &= -0.795 \frac{RT}{0.434 F} \log \frac{\sqrt{(\text{Na}^+)_2(\text{Cl}^-)_2}}{\sqrt{(\text{Na}^+)_1(\text{Cl}^-)_1}} \\ &= -0.795 \frac{RT}{0.434 F} \log \frac{\sqrt{[\text{Na}^+]_2[\text{Cl}^-]_2} \gamma_2}{\sqrt{[\text{Na}^+]_1[\text{Cl}^-]_1} \gamma_1} \end{aligned} \quad (2)$$

in which parentheses are used to denote activities and square brackets to denote concentrations.  $\gamma$  represents the mean activity coefficient for NaCl in the solution in question. It is assumed that the measurements of Fig 4 correspond to the average temperature, 22°C.

<sup>10</sup> As defined by Lewis and Randall<sup>11</sup> (page 327). Values of the activity coefficient for NaCl were obtained by graphical interpolation between values taken from a table compiled by H. S. Harned.<sup>12</sup> It is assumed that in these sea waters the activity coefficient for NaCl has the same value as in solutions of pure NaCl of the same ionic strength. Effects due to the presence of glycerol in the diluted sea waters are neglected.

<sup>11</sup> Lewis, G. N., and Randall, M. *Thermodynamics and the free energy of chemical substances*. New York: McGraw-Hill Book Co. Inc. 1923.

<sup>12</sup> Harned, H. S. in Taylor, H. S. *Treatise on physical chemistry*. New York: D. Van Nostrand Co., 2nd edition. 1931. 1, 772.

With the help of this equation, we may now calculate the variations in  $P D$  to be expected from the small differences in the activity of  $\text{NaCl}$  in the experiments described on pages 392 and 393. In the first of these experiments, the concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in the two solutions were identical, the activity coefficients for  $\text{NaCl}$ , however, were not the same, since the solutions differed considerably in ionic strength (0.24 and 0.42). Substituting in equation (2) the values of the activity coefficient for  $\text{NaCl}$  in 0.24 and 0.42 molar solutions (0.72 and 0.69), we find that a difference of 0.8 mv is to be expected.

In the second experiment, the concentration of  $\text{Cl}^-$  was the same in both solutions, the value of  $\gamma$  was also the same, since the solutions were of equal ionic strength. The concentrations of  $\text{Na}^+$ , however, were different, namely, 0.133 and 0.163 molar. Inserting these values in equation (2) we find that the  $P D$  with the former solution is expected to be the higher by 2.0 mv.

It will be seen that these calculated values are in excellent agreement with the experiments, confirming the assumption that only the  $\text{Na}^+$  and  $\text{Cl}^-$  ions are important in determining the  $P D$  with these diluted sea waters.

Although the high dilutions of  $\text{KCl}$ -sea water could not be included in Fig. 4 (because this sea water did not contain  $\text{Na}$ ), we may assume that there is no essential difference between the concentration effect with these solutions and the concentration effect with the dilute sea waters included in this figure. This assumption is borne out by the similar form of the  $P D$ -time curves and also by the similar slopes of the  $P D$ -dilution curves at higher dilutions. As shown in Fig. 2, these slopes increase regularly from curve  $D$  (natural sea water) to curve  $A$  ( $\text{KCl}$ -sea water). This change in slope, however, can be traced to the change in activity coefficient with dilution, when this factor is taken into account, as in Fig. 4, the difference in slope becomes insignificant. It is assumed, therefore, that in the concentration effect with all these solutions we are dealing with a single process—the outward diffusion of  $\text{Na}^+$  and  $\text{Cl}^-$  from the vacuole.

We may accordingly apply to the concentration effect with all these solutions the interpretation which was suggested in an earlier paper<sup>1b</sup> for the concentration effect with natural sea water. It is assumed that in all these cases we have to do with a diffusion potential, involving only the  $\text{Na}^+$  and  $\text{Cl}^-$  ions, in a non-aqueous layer of the protoplasm in which the mobilities of the ions may differ greatly from their values in aqueous solutions. We may assume that the layer in question is the outer surface (or  $X$ ) layer, judging from the promptness with which the  $P D$  arrives at its final constant value when the dilu-

tion of the external sea water is changed. In accordance with this interpretation, equation (2) is supposed to be equivalent to the familiar Nernst equation

$$P D_2 - P D_1 = \frac{u' - v}{u'' + v} \frac{RT}{0.434 F} \log \frac{c_2}{c_1} \quad (3)$$

Setting the factor,  $-0.795$ , of equation (2) equal to  $\frac{u'' - v}{u'' + v}$  gives us a new value for  $u''$ , the relative mobility of  $\text{Na}^+$  in the  $X$  layer. If  $v$ , the mobility of  $\text{Cl}^-$ , is arbitrarily taken as unity,  $u''$  is found to be  $0.114$ . (The difference between this and the earlier value,  $0.20$ , represents in part the effect of additional data, in part the effect of taking into account the change in activity coefficient with dilution.)

The quantities  $c_2$  and  $c_1$  in equation (3) are properly concentrations of  $\text{NaCl}$  in the non aqueous ( $X$ ) layer of the protoplasm in which  $u''$  and  $v$  are the mobilities of the ions. In place of these concentrations, which are unknown, equation (2) employs the mean activities of  $\text{NaCl}$  in the external sea waters. In assuming that equations (2) and (3) are equivalent, it is therefore implied that the concentrations of  $\text{NaCl}$  in  $X_0$  (the outer surface of the  $X$  layer) are proportional to these activities, i.e. that a distribution equilibrium has been set up with respect to  $\text{NaCl}$  and that the partition coefficient is constant. Evidently this was approximately true in the measurements with the dilute sea waters included in Fig. 4.

It is obvious however that as long as there is a net diffusion from one layer to the other, the two phases can at best be only approximately in equilibrium. With dilutions of sea waters containing still smaller concentrations of  $\text{NaCl}$  the deviations from equilibrium become too important to be neglected. For example, with the higher dilutions of the sodium free  $\text{KCl}$  sea water the concentration gradients in the  $X$  layer must have been practically the same as with equal dilutions of the sea water containing  $0.300$  molar  $\text{NaCl}$ , since (as shown in Fig. 2) approximately the same  $P D$ 's were observed with equal dilutions of the two sea waters. To calculate the  $P D$  in such cases would require a detailed knowledge of the diffusion process. Similarly the diffusion process must be taken into account in order to explain the failure of  $\text{K}^+$  to participate in the concentration effect at higher dilutions.

We have seen that the concentration effect with high dilutions of  $\text{KCl}$  rich sea waters is practically the concentration effect for  $\text{NaCl}$ .

alone, with  $K^+$  playing no significant part. As the dilution of the KCl-rich sea water is decreased, however, a value is reached at which  $K^+$  begins to influence the  $pD$ , and the  $pD$ -log dilution curve, as shown in Fig 2, begins to diverge from the straight line which represents the concentration effect for NaCl alone. The dilution at which this occurs will be called the critical dilution. With dilutions less than this critical value, the influence of  $K^+$  becomes increasingly important, finally bringing about the reversal of the sign of the concentration effect.

It is evident from Fig 2 that the greater the concentration of  $K$  in the undiluted sea water, the higher is the dilution required to prevent  $K^+$  from influencing the  $pD$ . A more exact knowledge of this relationship may be gained from a study of the diffusion process in the external non-aqueous ( $X$ ) layer of the protoplasm. In this study, we shall assume that  $X_0$ , the outer surface of this layer, has been brought into distribution equilibrium with the external solution in question, and investigate the movement of  $K^+$  ions in the ensuing diffusion process.

We may suppose that when the *Valonia* cell has been for a long time in natural sea water, the concentrations of  $K^+$ ,  $Na^+$ , and  $Cl^-$  will be uniform throughout the  $X$  layer, in distribution equilibrium with the external sea water, as shown in Fig 5a. When a cell is transferred from natural sea water to a diluted natural or KCl-rich sea water, we may imagine that in  $X_0$  distribution equilibrium with the new solution is set up at once, while in the main body of the  $X$  layer the concentrations remain for a time at their former values as in Fig 5a. Between these two regions there will be a concentration gradient, the length of which we may call  $\delta$ . Distances along this gradient normal to the boundary surface will be measured in terms of the linear coordinate  $x$ . This situation is illustrated in Fig 5b.

We may now inquire under what conditions ( $z e$ , with which external solutions)  $K^+$  will diffuse from this outer film further into the  $X$  layer, and under what conditions  $K^+$  will diffuse outward from the main body of the  $X$  layer into the surface film. Since it is probable that  $K^+$  will diffuse inward when the concentration of  $K$  in the external solution is very high, and outward when it is very low, the problem resolves itself into the calculation, for each of the KCl-rich sea

waters, of the critical dilution at which  $K^+$  does not diffuse in either direction in the  $X$  layer

Nernst<sup>13</sup> was the first to point out that the diffusion of an ion depends not only on the concentration gradient but also on the electrostatic forces which are set up in maintaining electro neutrality when

Natural sea water	$C_1'$	$C_1''$	$C_1$
Outer (X) layer of <i>Valonia</i> protoplasm	$c_1'$	$c_1''$	$c_1$
Main body of protoplasm			

FIG 5a

Diluted natural or KCl rich sea water	$C_2'$	$C_2''$	$C_2$
Outer (X) layer of <i>Valonia</i> protoplasm	$X_0$ $c_2'$	$c_2''$	$c_2$
Main body of protoplasm	$c_1'$	$c_1''$	$c_1$

$x = \delta$   
 $x = 0$

FIG 5b

FIG 5 Hypothetical diagrams of *Valonia* protoplasm, showing conditions which are assumed in the calculation of the critical dilutions. The symbols  $c_1'$ ,  $c_1''$ , and  $c_1$  denote the concentrations of  $K^+$ ,  $Na^+$ , and  $Cl^-$  in the outer, non aqueous (X) layer of the protoplasm, in distribution equilibrium with natural sea water, in which the concentrations of these ions are  $C_1'$ ,  $C_1''$ , and  $C_1$ . Similarly  $c_2'$ ,  $c_2''$  and  $c_2$  represent the concentrations of these ions in a portion of the X layer in distribution equilibrium with a diluted natural or modified sea water in which the concentrations of  $K^+$ ,  $Na^+$ , and  $Cl^-$  are  $C_2'$ ,  $C_2''$  and  $C_2$ .

ions have unequal mobilities. This has been expressed in mathematical form by Planck<sup>14</sup> in the equation

$$n' = -u RT \frac{dc'}{dx} - u e c' \frac{d\phi}{dx} \quad (4a)$$

where  $n'$  is the number of cations of some particular species (here  $K^+$ ) which at any point of the boundary layer pass through unit area in

<sup>13</sup> Nernst, W, *Z phys Chem*, 1888, 2, 613

<sup>14</sup> Planck, M, *Sitzungsber preuss Akad Wiss, physikal math Kl*, 1930, 367

unit time, the solvent being at rest. Similarly we may write for  $\text{Na}^+$

$$n'' = -u''RT \frac{dc''}{dx} - u''\epsilon c'' \frac{d\phi}{dx} \quad (4b)$$

and for  $\text{Cl}^-$

$$n = -vRT \frac{dc}{dx} + v\epsilon c \frac{d\phi}{dx} \quad (4c)$$

Here  $u'$ ,  $u''$ , and  $v$  represent the relative mobilities, and  $c'$ ,  $c''$ , and  $c$  the concentrations of the  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  ions in the  $X$  layer,  $R$ , the gas law constant,  $T$ , the absolute temperature,  $\epsilon$ , the charge on a gram mole of univalent ions,  $\phi$ , the electrostatic potential. If no current flows

$$n' + n'' = n \quad (5)$$

Substituting in equation (5) the values of  $n'$ ,  $n''$ , and  $n$  given in equations (4a, b, c), we obtain

$$\frac{d\phi}{dx} = -\frac{RT}{\epsilon} \frac{d/dx(u'c' + u''c'' - vc)}{u'c' + u''c'' + vc} \quad (6)$$

which is the fundamental equation for the diffusion potential as derived by Planck<sup>14</sup>

Eliminating  $\frac{d\phi}{dx}$  from equations (4a) and (6), we obtain an equation<sup>15</sup> for the number of diffusing  $\text{K}^+$  ions in terms of concentrations and mobilities alone

$$n' = -u'RT \left\{ \frac{dc'}{dx} - c' \frac{d/dx(u'c' + u''c'' - vc)}{u'c' + u''c'' + vc} \right\} \quad (7)$$

At the critical dilution, we assume that, as stated on page 398,  $\text{K}^+$  does not diffuse in either direction so that  $n' = 0$ . Then

$$\frac{dc'}{dx} = c' \frac{d/dx(u'c' + u''c'' - vc)}{u'c' + u''c'' + vc} \quad (8)$$

<sup>15</sup> Equations apparently similar to equation (7) but using a different notation are given by McBain, J. W., and Dawson, C. R., *J. Am. Chem. Soc.*, 1934, 56, 52

which may be simplified algebraically<sup>18</sup> to give

$$\frac{dc'}{dx} = c' \frac{d/dx(u'c - vc)}{u'c'' + vc} \quad (9)$$

Since  $u'$  does not appear in equation (9) we see that the critical dilution is independent of the mobility of the retarded ion

In order to integrate equation (9) the concentrations  $c$  and  $c''$  must be expressed as functions of  $x$ . For our purposes, however, it is not necessary to postulate any particular form of concentration gradient. Thus, for the concentration of  $\text{Cl}^-$  we may adopt the general expressions

$$c = c_1 + (c_2 - c_1)f(x) \quad (10)$$

$$\frac{dc}{dx} = (c_2 - c_1)f'(x) \quad (10a)$$

where  $f(x)$  may be any continuous function of  $x$  which satisfies the conditions

$$\text{when } x = 0 \quad f(x) = 0 \quad \text{and hence} \quad c = c_1$$

$$\text{when } x = \delta \quad f(x) = 1 \quad \text{and hence} \quad c = c_2$$

Here the subscripts have the same meaning as in Fig. 5. Equations similar to (10) may be written for  $c'$  and  $c''$ , using suitable functions of  $x$  (not necessarily identical with  $f(x)$ ). The three equations, however, must be related to one another in such a way that the law of electro-neutrality is maintained for all values of  $x$ . An expression for  $c''$  which fulfills this requirement is obtained by combining equation (10) with the equation expressing the condition of electro-neutrality  $c' + c'' = c$ . We get

$$c' = c_1 + (c_2 - c_1)f(x) - c \quad (11)$$

$$\frac{dc'}{dx} = (c_2 - c_1)f'(x) - \frac{dc}{dx} \quad (11a)$$

<sup>18</sup> By multiplying the equation by  $\frac{u'c + u'c'' + vc}{u'c + vc}$  then subtracting

$\frac{u'c'}{u'c + vc} \frac{dc'}{dx}$  from both sides

By substituting these values of  $c$  and  $c''$  in equation (9) and simplifying algebraically we obtain

$$\frac{1}{c'} \frac{dc'}{dx} = \frac{u'' - v}{u'' + v} (c_2 - c_1) \frac{f'(x)}{c_1 + (c_2 - c_1)f(x)} \quad (12)$$

which may now be integrated over the length of the concentration gradient

$$\int_{c_1'}^{c_2'} \frac{dc'}{c'} = \frac{u'' - v}{u'' + v} (c_2 - c_1) \int_0^{\delta} \frac{f'(x)dx}{c_1 + (c_2 - c_1)f(x)} \quad (13)$$

to give the expression

$$\log \frac{c_2'}{c_1'} = \frac{u'' - v}{u'' + v} \log \frac{c_2}{c_1} \quad (14)$$

which characterizes the critical dilution

Before equation (14) can be used, however,  $c_1$ ,  $c_2$ ,  $c_1'$ , and  $c_2'$ , representing unknown concentrations in the  $X$  layer, must be expressed in terms of known concentrations or activities in the external solution with which the  $X$  layer is assumed to be in distribution equilibrium. The assumptions and approximations involved in this substitution are brought out in the following discussion.

The activity<sup>17</sup> of KCl in a  $d$ -fold dilution of natural or KCl-rich sea water is

$$a_w = k_1 \frac{C_K C_{Cl}}{d^2} \gamma_{KCl}^2 \quad (15)$$

where  $C_K$  and  $C_{Cl}$  are the total concentrations of K and Cl in the *undiluted* sea water and  $\gamma_{KCl}$  is the mean activity coefficient for KCl in a sea water of dilution  $d$ . (For strong electrolytes, like KCl in aqueous solution, it is customary to define the activity in such a way that  $k_1$  is equal to unity.) For the activity of KCl in  $X_0$  we may write

$$a_x = k_2' c' c \quad (16)$$

assuming that in this layer we are dealing with such dilute solutions that the concentrations of the ions may be substituted for their activities without introducing serious error. Here, as in Fig. 5,  $c$  and  $c'$  represent concentrations of  $Cl^-$  and  $K^+$  ions—not total concentrations of Cl and K—in the  $X$  layer. Since

<sup>17</sup> As defined by Lewis and Randall<sup>11</sup> and represented in their notation by the symbol  $a_2$



$\lambda_0$  is in distribution equilibrium with the external solution,  $a$  will be proportional to  $a_w$ , then

$$c \ c = k_2 \frac{C_K C_{Cl}}{d^2} \gamma_{KCl}^{\frac{1}{2}} \quad (17a)$$

Similarly we may write for the distribution of NaCl between the same two phases

$$c \ c = k_2 \frac{C_{Na} C_{Cl}}{d^2} \gamma_{NaCl}^{\frac{1}{2}} \quad (17b)$$

In the present calculations, we may neglect the small difference between the activity coefficients for KCl and NaCl, and use the same value of  $\gamma$  for both salts. For this value we shall take the mean of  $\gamma_{KCl}$  and  $\gamma_{NaCl}$ , the activity coefficients for KCl and NaCl in solutions of the pure salts having the same ionic strength as the  $d$  fold dilution of sea water.

In equations (17a) and (17b),  $k_2$  and  $k_2''$  represent the distribution coefficients for KCl and NaCl. Adding these two equations and introducing for convenience the ratio,  $r$ , defined by equation (18)

$$r = \frac{k_2}{k_2''} \quad (18)$$

we obtain

$$c(c + c) = \frac{k_2 \gamma^{\frac{1}{2}}}{d^2} C_{Cl}(C_{Na} + r C_K) \quad (19)$$

If KCl and NaCl are the only substances present in the  $\lambda$  layer which furnish  $K^+$ ,  $Na^+$ , and  $Cl^-$  ions in significant amounts

$$c + c = c \quad (20)$$

and

$$c = \frac{k_2 \gamma}{d} \sqrt{C_{Cl}(C_{Na} + r C_K)} \quad (21)$$

Corresponding expressions for the concentrations of  $Na^+$  and  $K^+$  in  $\lambda_0$  are obtained by dividing equation (17a) by (17b) and introducing (18) and (20). For  $K^+$  this gives us

$$c = \frac{r C_K c}{C_{Na} + r C_K} \quad (22)$$

To calculate the critical dilutions of the various KCl rich sea waters, we may now combine equations (21) and (22) with (14) and substitute numerical values. We obtain, after rearrangement, an equation

$$\log \frac{d^*}{\gamma d} = \log \frac{1}{\gamma_1} + \frac{u}{2v} \log \frac{C_K}{0.012} + \frac{u}{2v} \log \frac{0.488 + 0.012 r}{C_{Na} + r C_K} \quad (23)$$

which tells us (if the value of  $r$  is known) the value of  $\frac{d^*}{\gamma_d}$  for a KCl-rich sea water in which the concentrations of potassium and sodium are  $C_K$  and  $C_{Na}$ . Here  $d^*$  represents the critical dilution,  $\gamma_d$ , the activity coefficient for KCl and NaCl in sea water of this dilution,  $\gamma_1$  ( $= 0.64$ ), the activity coefficient for KCl and NaCl in undiluted sea water, 0.012 and 0.488 the molar concentrations of K and Na in ordinary sea water,  $u''$  ( $= 0.114$ ), and  $v$  ( $= 1.00$ ), the relative mobilities of  $Na^+$  and  $Cl^-$  in the  $X$  layer,  $r$ , the ratio defined in equation (18). The value of the critical dilution,  $d^*$ , can readily be obtained from  $\frac{d^*}{\gamma_d}$  by means of a graph<sup>18</sup> in which  $\frac{d}{\gamma}$  is plotted as a function of  $d$ .

Before  $\frac{d^*}{\gamma_d}$  can be calculated, however, some assumption must be made as to the value of  $r$ , the ratio of the distribution coefficients for KCl and NaCl. For an analogous case, Shedlovsky and Uhlig<sup>19</sup> have predicted from theoretical considerations that the smaller the ion sizes the smaller will be the partition coefficients (defined as in equations (17a, b)). This prediction is confirmed by their measurements of distribution between water and guaiacol. Since the ionic radius of  $Na^+$  is less than that of  $K^+$ , we may assume in accordance with their prediction that  $k_3''$  is smaller than  $k_3'$ , and hence that  $r$  is not less than unity.

We then find that according to equation (23) it makes little difference what value (greater than unity) is assigned to  $r$ . If as a lower limit we set  $r$  equal to unity the final term of equation (23) becomes zero, since in all these sea waters  $C_{Na} + C_K = 0.500$ . If, on the

<sup>18</sup> Data for constructing such a graph are given in the following table. The values of  $\gamma$  given in the second line are means of  $\gamma_{KCl}$  and  $\gamma_{NaCl}$  taken from the table compiled by Harned<sup>12</sup>. The third line gives the dilutions of Bermuda sea water which have the ionic strengths listed in line 1.

Ionic strength	0.05	0.1	0.2	0.5	1.0
Activity coefficient, $\gamma$	0.818	0.771	0.722	0.661	0.627
Dilution, $d$	14.43	7.23	3.61	1.44	0.72
$\frac{d}{\gamma}$	17.64	9.38	5.01	2.18	1.15

<sup>19</sup> Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933-34, **17**, 563.

other hand, we allow the value of  $r$  to increase without limit, the last term of equation (23) approaches  $\frac{u''}{2v} \log \frac{0.012}{C_K}$ . The following table gives the values of the critical dilution for the sea waters used in these experiments, calculated for these two extreme cases. It will be seen that the calculated values of  $d^*$  are little influenced by the value assigned to  $r$ .

Molar concentration of K in undiluted sea waters	0.500	0.200	0.050	0.012
Critical dilution, calc., if $r$ is equal to unity	10.0	5.7	2.4	1.0
Critical dilution calc., if $r$ is infinitely large	7.9	4.8	2.3	1.0

In the case of natural sea water, equation (23) states that the undiluted solution represents the critical dilution. This follows directly from the assumption (Fig. 5a) of uniform concentrations of  $K^+$ ,  $Na^+$ , and  $Cl^-$  throughout the  $X$  layer in distribution equilibrium with natural sea water, since in the absence of concentration gradients we should not expect a net diffusion in either direction.

The assumption that the concentrations of  $K^+$ ,  $Na^+$ , and  $Cl^-$  are uniform throughout the  $X$  layer when the cell has been for a long time in natural sea water does not take into account the fact that cells growing in ordinary sea water actually do take in  $KCl$  and  $NaCl$ . Accordingly, these calculations apply strictly only to cells which are not growing. In the case of growing cells, however, the calculations may be regarded as approximately correct, since we may assume that the net diffusion which accompanies growth in ordinary sea water is a very slow process in comparison with the diffusion which follows the important changes in the composition of the external solution during the experiments reported here.

Experiments show that the critical dilution for natural sea water is in fact somewhat greater than the calculated value, unity. If the  $K^+$  ions present in ordinary sea water were actually without influence, the  $P.D.$  should remain unchanged when these ions are replaced by  $Na^+$  ions. It has been found,<sup>20</sup> however, that the  $P.D.$  with such  $KCl$  free sea water is 2 to 4 mv. less than with ordinary sea water.

<sup>20</sup> Damon, E. B. *J. Gen. Physiol.*, 1932-33, 16, 378, curve marked  $C_K = 0$  also unpublished measurements.

This effect is much too large to be ascribed to the change in concentration of  $\text{Na}^+$  alone, which according to equation (16) should lead to a decrease of only 0.2 mv. On the other hand, the effect is considerably less than might be predicted from the behavior with KCl-rich sea waters. It has been shown<sup>21</sup> that the initial rise in  $P D$  with undiluted KCl-rich sea waters is given by the expression

$$P D = 59.1 \log (BC_K + D) \quad (\text{mv at } 25^\circ) \quad (24)$$

where the constants  $B$  and  $D$  have the values 23.4 and 0.72, respectively. According to this equation, a decrease of 8.5 mv is to be expected with KCl-free sea water. We may conclude, accordingly, that while  $\text{K}^+$  still plays a part in the  $P D$  with ordinary sea water, its influence is diminished because this solution is not far from the critical dilution, a slight dilution then suffices to prevent  $\text{K}^+$  from affecting the  $P D$ . This is the behavior which we should expect in growing cells.

In the case of the KCl-rich sea waters, the variation in the observed  $P D$ 's is too great to permit assigning precise values to the critical dilutions. It will be seen from Fig. 2, however, that the range of calculated values given in the above table is consistent with the experiments. The agreement between the observed and calculated values of critical dilution is especially striking if the  $P D$ -dilution curves of Fig. 2 are fitted to the highest (instead of the average) observed values of  $P D$ . Since in these measurements the experimental errors tend to make the observed  $P D$ 's too low, there is good reason to believe that these highest values of  $P D$  are the most reliable.

It is evident from Fig. 2 that the observed values of critical dilution with the KCl-rich sea waters are in no case higher than the values computed for  $r = 1$ . This confirms the assumption, based on the prediction of Shedlovsky and Uhlig, that the distribution coefficient for KCl is not less than that for NaCl. Unfortunately, we cannot draw from the present data a more definite conclusion as to the value of  $r$ . It is possible that  $r$  may vary considerably from cell to cell, or in the same cell under different conditions.

The success of these calculations of critical dilution shows the importance of the diffusion process in the  $X$  layer for determining the

<sup>21</sup> Damon, E. B., *J. Gen. Physiol.*, 1932-33, 16, 392

behavior of  $K^+$  in the concentration effect With those smaller dilutions of KCl rich sea waters where  $K^+$  plays an important part in the P.D., we find that the diffusion of  $K^+$  in the  $X$  layer is directed *inward*  $K^+$  enters the protoplasm from these solutions With higher dilutions of these sea waters, where  $K^+$  appears to play no part in the P.D., we find that the diffusion of  $K^+$  in the  $X$  layer is directed *outward*  $K^+$  tends to pass from the protoplasm to the external solution until the concentration gradient in  $X$  reaches the steady state defined by equation (14) Since the P.D. with these solutions shows no evidence of the outward diffusion of  $K^+$  we may conclude (1) that the amount of KCl ordinarily present in the protoplasm is too small to produce any lasting electrical effect when the external sea water is diluted and (2) that under the conditions of these experiments KCl does not diffuse out from the vacuole rapidly enough to affect the P.D. appreciably

In contrast to the behavior of  $K^+$ , it appears that there is little hindrance to the outward diffusion of  $Na^+$  and  $Cl^-$  from the vacuole From the constancy of the P.D. in dilute sea waters (above the critical dilution) we may infer (1) that NaCl comes out from the vacuole rapidly enough to maintain a practically constant concentration gradient in the  $X$  layer, and (2) that the outward diffusion of NaCl has little effect on the P.D. at the  $Y$  layer It is possible that small changes in the P.D. at  $X$  are compensated by simultaneous changes in the P.D. at  $Y$

The failure of  $K^+$  to come out from the vacuole indicates that the mechanism by which KCl is accumulated in the cell sap remains effective when the cell is exposed for brief intervals to isotonic dilutions of sea waters Unfortunately, the study of concentration effect has furnished no new information as to the nature of this mechanism In the present discussion, it has been tacitly assumed that this mechanism is to be found at the  $Y$  layer It may consistently be assumed, for example, that the  $Y$  layer behaves like the guaiacol layer of the model proposed by Osterhout and his coworkers<sup>22</sup>

It has been mentioned in earlier reports that more drastic treatment (too prolonged exposure, or exposure to too high dilutions) produces in *Valonia* secondary changes which are made evident by a sudden

<sup>22</sup> Osterhout, W J V *Ergebn Physiol*, 1933, 35, 988

reversal of the sign of the  $P D$  across the protoplasm. It is perhaps significant that this change in  $P D$  is in the direction which we should expect from outward diffusion of  $KCl$  from the vacuole, if this were made possible by a breakdown of the mechanism by which  $KCl$  is accumulated.

Since this reversal of the sign of the  $P D$  is produced also by exposure to undiluted  $KCl$ -free sea water<sup>30</sup>, it appears that the decreased activity of  $KCl$  in the external solution, rather than any other effect of dilution, may be the most important factor in causing the secondary changes. This would explain why greater dilutions of  $KCl$ -rich sea waters than of natural sea water can safely be applied to *Valonia*. In the dilutions of  $KCl$ -rich sea waters included in Fig. 2, for example, the activity of  $KCl$  is in all cases greater than in the fivefold dilution of natural sea water.

#### SUMMARY

The concentration effect with sea waters containing more than the normal amount of potassium has been studied in *Valonia macrophysa*. This was done by comparing the initial changes in  $P D$  across the protoplasm when natural sea water bathing the cell was replaced by various isotonic dilutions of  $KCl$ -rich sea waters.

With small dilutions of  $KCl$ -rich sea waters, the  $P D$ -time curves are of the same form as with the undiluted solution, exhibiting the fluctuations characteristic of  $KCl$ -rich solutions. This indicates that with these solutions  $K^+$  enters *Valonia* protoplasm and plays an important part in the  $P D$ . The value of the initial rise in  $P D$  decreases with increasing dilution.

With high dilutions of  $KCl$ -rich sea waters, the  $P D$ -time curves are of quite different shape, resembling the curves with diluted natural sea water, the  $P D$  is practically independent of small changes in the concentration of potassium, and increases with increasing dilution. That is, with these higher dilutions, the sign of the concentration effect is reversed, becoming the same as with diluted natural sea water.

The greater the concentration of  $KCl$  in the undiluted sea water, the higher is the critical dilution at which  $K^+$  ceases to influence the  $P D$ .

For a wide range of sea waters containing both KCl and NaCl, it is shown that the concentration effect above the critical dilution is determined solely by the activity of NaCl in the external solution. It is concluded that with dilute natural sea water and with high dilutions of KCl rich sea waters we have to do with a diffusion potential, involving only the  $\text{Na}^+$  and  $\text{Cl}^-$  ions, which are diffusing out from the vacuole.

A quantitative relation between the composition of the sea water and the critical dilution has been deduced from the classical theory of the diffusion of electrolytes. It is shown that with dilutions less than this critical value the diffusion of  $\text{K}^+$  in the outer non aqueous layer of the protoplasm is directed inward, hence  $\text{K}^+$  enters the protoplasm from these solutions. With dilutions greater than the critical value, the diffusion of  $\text{K}^+$  in this layer is directed outward, hence  $\text{K}^+$  does not enter the protoplasm.

Since the p.d. shows no evidence of this outward diffusion of  $\text{K}^+$ , it is concluded that the amount of  $\text{K}^+$  ordinarily present in the protoplasm is too small to produce any lasting electrical effect, and that the outward diffusion of  $\text{K}^+$  from the vacuole is prevented by the mechanism responsible for the accumulation of KCl in the cell sap.





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# THE ABSORPTION SPECTRUM OF VISUAL PURPLE

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## I

### *Classical and Unbleached Absorption Spectrum*

The classical absorption spectrum of visual purple (Kottgen and Abelsdorff, 1896) is the difference in photometric density ( $\log I_0/I$ ) at different wave lengths between a solution in its unbleached and its bleached condition. The reason for this procedure is that extracts of visual purple are not pure but contain other substances which may be colored. The resulting classical subtraction curve is a nearly symmetrical absorption spectrum with a maximum at about 500  $m\mu$ . It undoubtedly represents an important property of visual purple, because it resembles closely the rod visibility curve (Hecht and Williams, 1922, Weaver, 1937, Dartnall and Goodeve, 1937).

The subtraction procedure is valid only if visual purple is the one light sensitive substance in the retinal extract, if no new color appears when visual purple is bleached, and if the absorption of the photolabile group in the molecule is solely responsible for its color. It is not certain that visual purple is the only light sensitive substance in the frog's retinal extract (Chase, 1937), and it has been repeatedly observed that under some conditions a new color arises when visual purple is bleached (Kühne, 1879, Garten, 1907, Nakashima, 1929, Weigert and Nakashima, 1930, Hosoya, 1933, Wald, 1935, Chase, 1936, Lythgoe, 1937, and others). Finally, there is no experimental justification for the assumption that the molecule aside from its light sensitive group or groups is colorless. The protein nature of the molecule (Kühne, 1879, Wald, 1935, and Hecht, Chase, and Shlaer, 1937) makes the third assumption even less tenable because proteins absorb some light in the visible region of the spectrum.

If the solutions could be freed of other colored substances, then the



Fig 1 shows difference curves for solutions prepared in a variety of ways presently to be described, their densities at 500 m $\mu$  were made equal to 1.0. The solid line in Fig 1 is the average of the five sets of data within the envelope. It is very similar to Kottgen and Abelsdorff's classical absorption spectrum, also shown in the figure. Its 500/420 ratio is 8.0. In no case has this ratio for unbleached visual purple solutions been greater than 2.8, seldom as high, and usually considerably less.

In the effort to make the absorption of the unbleached solution resemble the classical absorption spectrum, we have subjected the retinas and the retinal extracts to various treatments designed to eliminate absorption by substances other than visual purple. The extreme lability of visual purple prohibits the methods of chemical purification usually employed in such a situation and has limited us to the use of a few physical and chemical processes. Since at present the only criterion of visual purple purity is its color, all analyses have been made with the sensitive photoelectric spectrophotometer designed by Schlaer (1938).

## II

### *Methods of Preparation*

(A) *Procedure*—The method commonly employed for extracting visual purple from the frog's retina is essentially that used by Kühne (1879). A number of frogs (*Rana pipiens*, freshly received from Alburg, Vermont, in our case) are dark adapted overnight at room temperature. The heads are then cut off and rinsed in water or physiological salt solution and the eyes removed. These are cut through just back of the iris with a razor blade, the lens discarded and the retina lifted out from the bulbus and put in salt solution or distilled water. After all of the retinas have been collected in this way, they are centrifuged and the supernatant liquid discarded. A quantity of 4 per cent purified bile salts solution 2 per cent digitalin,<sup>1</sup> or other extractive is then added in the proportion of about 1 cc. for each 25 retinas and the extraction allowed to proceed for the desired time, 10 minutes to 2 hours, with or without stirring and at any desired temperature. The mixture is then centrifuged at a fairly high speed for  $\frac{3}{4}$  hour to  $1\frac{1}{2}$  hours, usually at 6°C. in our case since low temperature retards decomposition during centrifugation. The resulting liquid is carefully pipetted off from the packed retinal debris and pigment and stored at 0-6°C. until it is used, which is usually within 48 hours after preparation.

<sup>1</sup> Digitalin crystalline, lot G57, Eimer and Amend, New York.

The solutions we have prepared in this way, as well as those so prepared by most other investigators, are invariably slightly turbid, exhibit a Tyndall cone, and absorb strongly in the blue-violet. Hardening the posterior half of the eye in 4 per cent alum solution before the retina is removed greatly decreases the blue-violet absorption of the resulting unbleached solution and also decreases its turbidity. Experiment has shown that 2 hours is the optimum time for the effect. The alum must be carefully washed out, especially if the pH of the visual purple solution is to be changed.

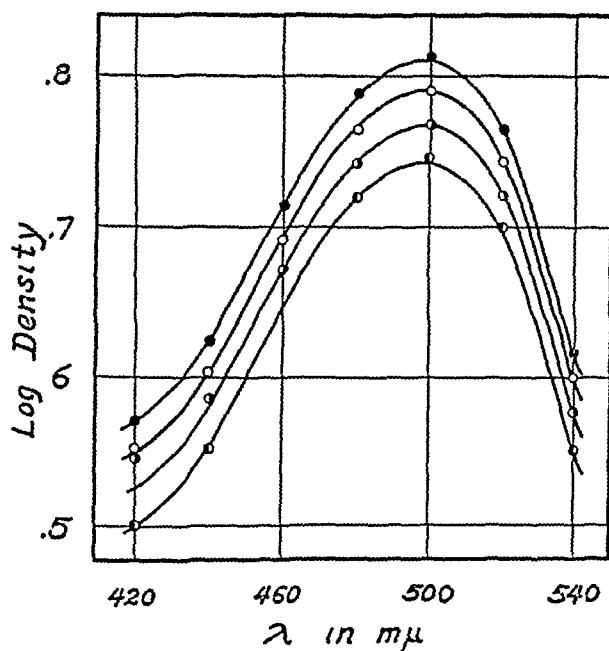


FIG. 2. Four simultaneous extractions made under identical conditions. The data, plotted on an arbitrary scale as log density, are shifted at 500  $m\mu$  so as to be equally separated from each other. The same curve is drawn through the four sets of data. It is apparent that at all wave-lengths the four curves are equidistant from each other, with the exception of two points. This is proof that these four simultaneous extractions contain the same relative amounts of all constituents and that differences experimentally produced in such simultaneous extractions are significant.

(B) *Normal Variation*—Before any critical experiments could be undertaken to test the effect of different treatments on the visual purple absorption spectrum, it was necessary to evaluate the normal range of variation in identical extractions.

Four simultaneous extractions were made under identical conditions from the retinas of equal numbers of frogs dark adapted at the same

time Alum was used to harden the retinas and 2 per cent digitalin to extract them, and the solutions were afterwards buffered at pH 7.76. The density values of the four extractions at  $500\text{ m}\mu$  varied in a random way by about 20 per cent. After subtraction of the density of the cell filled with water, log density of the four unbleached solutions was plotted against wave length.<sup>2</sup> In Fig. 2 these values are shown, after shifting the curves vertically to separate the points at  $500\text{ m}\mu$  from each other by the same equal distance. With the exception of two points, this results in an equal separation of all the points at all wave lengths, which shows that the four extractions contain the same relative proportions of all constituents, although their absolute densities are different.

In view of this identity, any differences that appear as the result of treatment during an extraction can be considered significant.

### III

#### *Effects of Various Treatments*

(A) *Alum*—Fig. 3 shows the absorption spectra of unbleached solutions prepared with and without alum pre-treatment. The densities have been made equal to 1.0 at  $500\text{ m}\mu$ , after allowing for the cells' reflection and absorption. Clearly, the alum treatment increases the transmission of the solution in the blue and violet. It is possible that the apparent extra absorption when alum has not been used is really dispersion and scattering of light, and the following considerations bear this out.

The absorption spectra of the purest unbleached extractions prepared with alum are almost identical with the classical difference curve of Fig. 1 between  $600$  and  $500\text{ m}\mu$ . Consequently the subtraction of the difference curve leaves a residual density curve which increases from zero at  $500\text{ m}\mu$  to an appreciable value at  $420\text{ m}\mu$ . This curve is not describable throughout the spectrum by the Rayleigh equation.

<sup>2</sup> In all cases where data are plotted as log density, an arbitrary logarithmic scale has been used since the values can then be shifted vertically. The absolute density of most of the solutions tested was slightly less than 1.0 for 10 millimeters depth measured at  $500\text{ m}\mu$ .

for scattering of light,<sup>3</sup> and consequently represents real light absorption rather than dispersion

In the case of an extraction made *without* alum, on the other hand, subtraction of the classical difference curve leaves a residual density

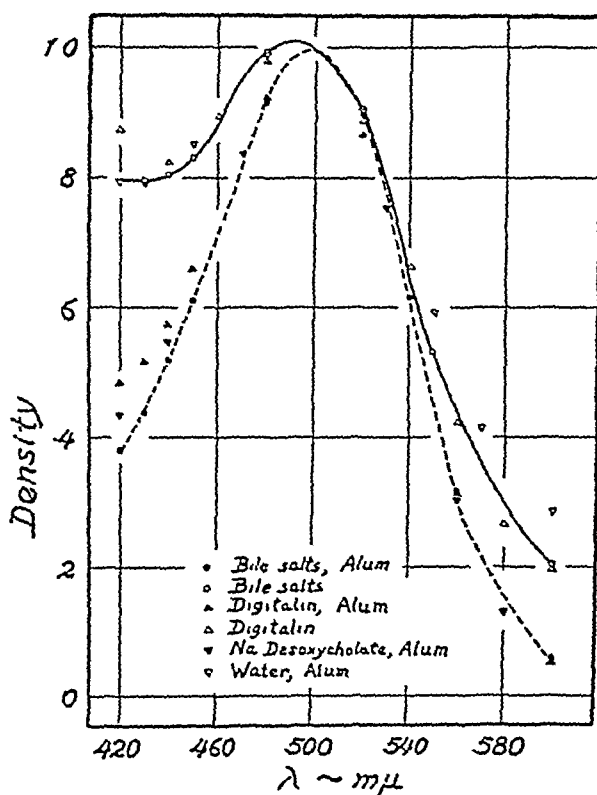


FIG 3 Visual purple solutions prepared with various extractives with and without the use of alum. The solid line is drawn through the data from the non-alum-treated bile salts extraction, and the broken line through the data from the alum-treated bile salts extraction. Note that the use of alum in the extraction caused a generally decreased density throughout the spectrum, except in the case of the water extraction. All data have been adjusted to have a density of 1.0 at 500  $m\mu$ .

curve which has a low value at 600  $m\mu$ , increases gradually to 500  $m\mu$ , and then more rapidly to 420  $m\mu$ . When these residual density

<sup>3</sup>  $I = I_0 e^{-K\lambda^{-4}z}$ , where  $z$  is the thickness of the scattering medium and  $K$  is a constant characteristic of the solution (Strutt, J. W., 1871). For convenience the equation can be thrown into its linear form and density can then be plotted against  $1/\lambda^4$ .



values are tested with the Rayleigh equation, the fit is good from 600 to 500  $m\mu$  but not from 500 to 420  $m\mu$ . Therefore both the alum and non alum extractions contain a substance which absorbs light of wave lengths shorter than 500  $m\mu$ , and the non alum extractions contain in addition a light dispersing factor. The unbleached absorption spectrum of a non alum extraction should then be capable of separation into at least three components, the light absorption of the photosensitive group (probably represented by the classical visual purple absorption spectrum), the light absorption caused by a yellow light stable group or substance, and a light dispersing factor not present in alum extractions.

The classical visual purple absorption spectrum can be calculated from the measurements of the unbleached and bleached non alum solution. The absorption spectrum of the light stable component, however, must be obtained in a less direct way. If this light stable component is in the visual purple molecule itself, its density should be proportional to that of the light sensitive group. On this assumption the absorption spectrum of the light stable group in the non alum extraction can be found by multiplying its absorption spectrum in the alum extraction (where no complicating dispersion factor is present) by the ratio between the densities of the light sensitive groups in the two types of extraction.

Having obtained the absorption spectrum of the light stable yellow component of the non alum extraction in this way, it can be subtracted, along with that of the photosensitive group, from the absorption spectrum of the unbleached non alum extraction. When this is done and the resulting density values are plotted against  $1/\lambda^4$ , a straight line describes the values throughout the visible spectrum. Therefore, visual purple extractions made without hardening the retinas with alum appear to differ from those in which alum is used merely by dispersion of the incident light, that is, by the presence of substances in the solution which scatter rather than absorb the beam. The fact that the residual density curve calculated for the purest alum extractions is not described by the dispersion equation indicates that in this case a true light absorption takes place, probably representing the photostable part of the visual purple molecule.

(B) *Re extraction*—This conclusion is supported by experiments on repeated extractions. Three successive 10 minute extractions were

made on the same batch of retinas, using 2 per cent digitalin solution at 0°C. The absolute density values at 500 m $\mu$  were respectively, 1.045, 0.355, and 0.275, in a 10 millimeter cell, after subtracting the density of the cell and water.

For purposes of comparison, log density values were computed for the three curves and each curve was shifted on the log density axis

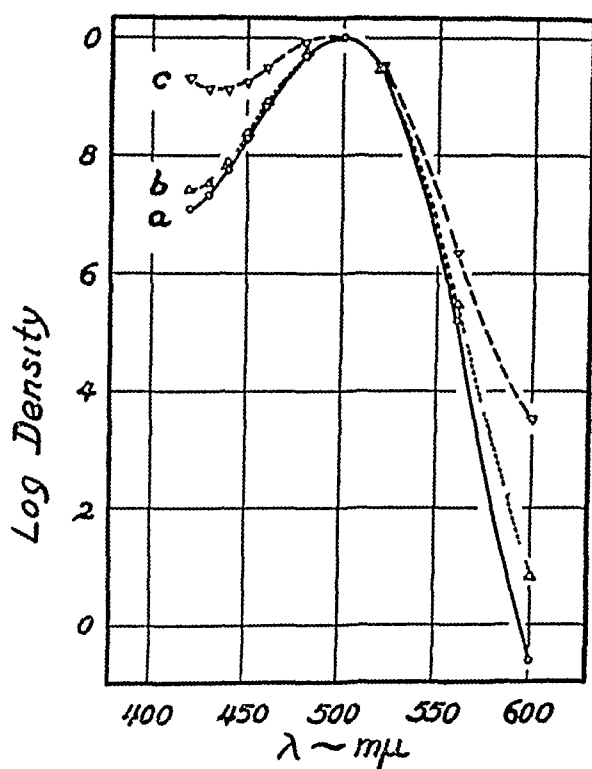


FIG. 4. Three successive extractions with 2 per cent digitalin on the same batch of retinas, alum-treated. The data have been plotted as log density on an arbitrary log scale after having been adjusted to be equal at 500 m $\mu$ . Note that curve *a*, that of the first extraction, shows relatively more visual purple color than does curve *b*, the second extraction, or curve *c*, the third.

so that the points at 500 m $\mu$  are superimposed. Fig. 4 shows that the first extraction contains relatively more visual purple than the two succeeding ones. These results indicate that a great deal of the color, particularly that resulting from absorption of blue and yellow, is

caused by extraction of substances other than visual purple, and which are less soluble in 2 per cent digitalin solution

(C) *Extractives*—Simultaneous extractions were made from alum treated retinas under identical conditions, using aqueous solutions of 2 per cent digitalin, 4 per cent purified bile salts, 4 per cent sodium desoxycholate solution,<sup>4</sup> and distilled water, and were buffered to pH 9.2 Fig 5 shows that the absorption spectra of these four un

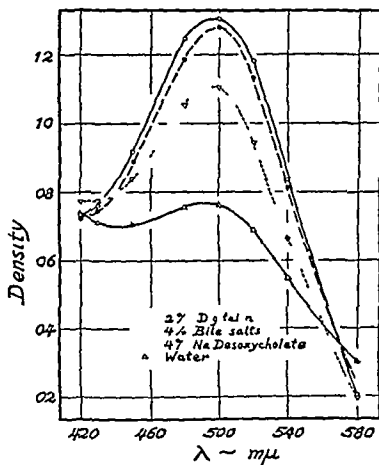


FIG 5 Unbleached visual purple absorption spectra of four simultaneous extractions made with different substances. The original data have been plotted after subtraction of the reflection and absorption of the cell filled with water. Alum was used to harden the retinas. The water extraction contains relatively less visual purple color than do the others.

bleached solutions are similar except for the water extraction, which gives a relatively high absorption in the blue and yellow. In Fig 6 the difference spectra of these extractions are plotted and the average of the four sets of data shown by the solid line. Evidently the clas

<sup>4</sup> Riedel de Haen Inc., New York

sical difference spectrum is not affected by the kind of extractive used, although the absorption of the solution as a whole is modified considerably, especially in the case of the water extraction. Very likely the difference spectrum records the absorption of the light-sensitive component of the molecule more nearly than do the spectra of the unbleached solutions.

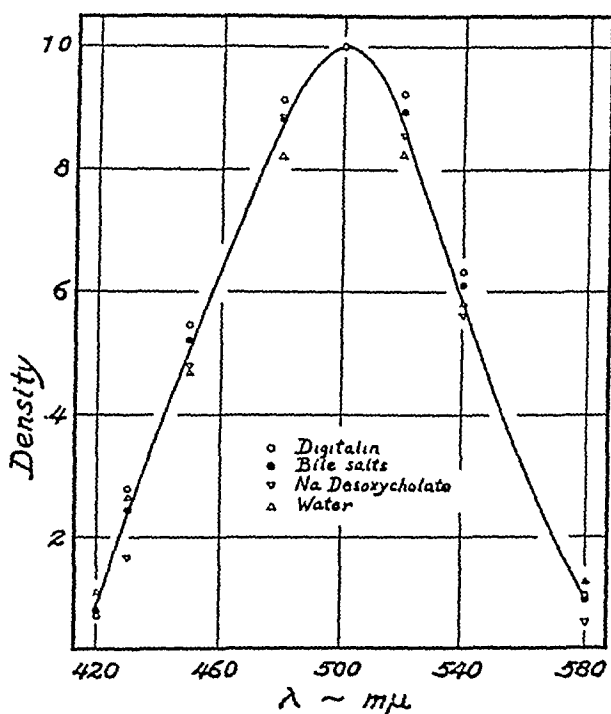


FIG. 6 Classical visual purple absorption spectra calculated from the curves shown in Fig. 5 and from the curves of the bleached solutions (not shown). All values are adjusted so as to have a density of 1.0 at 500  $m\mu$ . The solid line is the average of the four curves. Note that the classical difference curve is not affected by the kind of extractive.

Sodium desoxycholate extractions are unique in that no regeneration of visual purple occurs after bleaching. Addition of 4 per cent of sodium desoxycholate to a bile salts or digitalin extraction of visual purple buffered at pH 7.76 also prevents regeneration (Hecht, Chase, Shlaer, and Haig, 1936).

Hosoya used several different extractives: digitonin, sodium glycocholate, sodium oleate, saponin, panaxtoxin, and sodium salicylate,

but without alum pre treatment (Hosoya, 1933, Hosoya and Bayerl, 1933) As is to be expected, the solutions all show a high density in the blue, with a low 500/420 ratio which is frequently less than 1 The extractives vary among themselves in effectiveness, sodium salicylate being the worst

Our water extracts are apparently much like those produced by the other extractives and agree with Dartnall and Goodeve's findings (1937) Kubne (1879) could not make any water extracts, and suggested that such apparent extracts are fine suspensions of rod fragments However, microscopic examination after addition of concentrated NaCl to our extracts has not shown such fragments to be present The visual purple color rapidly disappears from such extractions even in the dark at 0°C, and it seems likely that the presence of bile salts, digitalin, or some other substance has a protective effect on the visual purple molecule in solution This is borne out by experiments with different concentrations of extractive

(D) *Digitalin Concentration*—Three simultaneous extractions were made from one batch of retinas, using 3, 6, and 10 per cent digitalin solution The log density plots of Fig 7 show that the digitalin concentration probably does not influence the visual purple purity within the concentration range tested, nor does it affect the density of the extract very much But it does influence its keeping power

After measurement, these three solutions were stored in the dark at 0° for 7 weeks and then measured again Fig 8 shows that the higher the digitalin concentration, the less the decrease in density at 500 mμ during this period, indicating that the digitalin protects the visual purple

(E) *Aging*—The decrease in visual purple concentration, described in the preceding section, occurs much faster at room temperature in both 4 per cent bile salts and 2 per cent digitalin extractions

Measured at 500 mμ a density decrease always occurs Its rate is least around pH 7.6 The  $Q_{10}$  for this change is approximately 8, indicating that the phenomenon may be due to heat denaturation of a protein Kuhne long ago came to the same conclusion (1879) reiterated recently by Mirsky (1936)

The density of bile salts extractions decreases at all wave lengths Digitalin extractions, however show in alkaline reactions an increase

in density in the blue and a decrease in other parts of the spectrum, while in acid reactions they behave like the bile salts extractions

The 500/420 density ratio of the unbleached visual purple absorption spectrum is never improved by these changes, the fresh solutions always show a more symmetrical curve

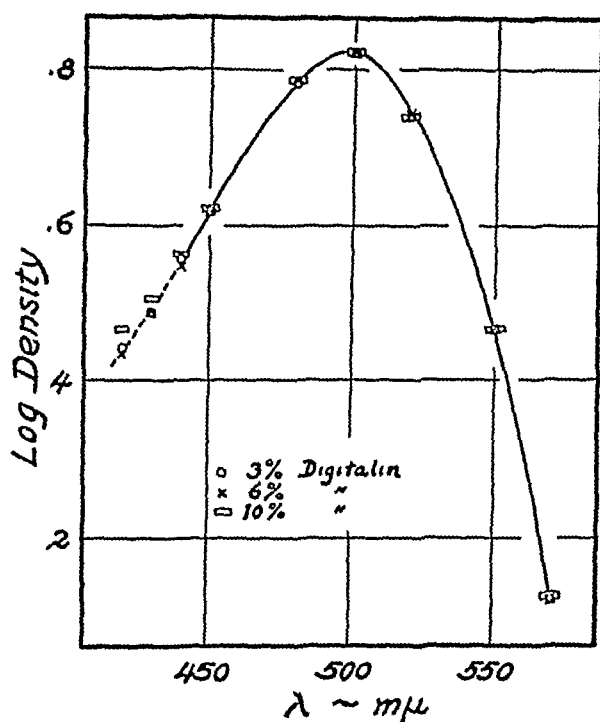


FIG 7 Simultaneous extractions made with three concentrations of digitalin. Log density is plotted on an arbitrary scale after superimposing the points at 500 mμ. The concentration of digitalin has very little effect on the relative amounts of retinal substances extracted, except perhaps in the case of the 10 per cent digitalin extraction where the purity of visual purple color is slightly less.

(F) *Hydrogen Ion Concentration* — Visual purple is a protein, and if other proteins are present, their isoelectric points are probably not the same, and different pH's might affect them differently and thus influence the solutions of visual purple. We first tested the influence of pH after extraction, and then during extraction.

A solution of visual purple extracted at 30° for 10 minutes was divided into five samples, and buffered to pH 5.50, 5.76, 6.63, 7.78, and 10.08 respectively. The absorption spectra of the unbleached samples are

plotted in Fig 9 as log density with all the points at  $500\text{ m}\mu$  superimposed for comparison. They show that there is a regular, but very slight, increase in symmetry as the pH increases. This may mean that the unbleached visual purple molecule contains some group which behaves as an indicator for  $[\text{H}^+]$ , as its decomposition product

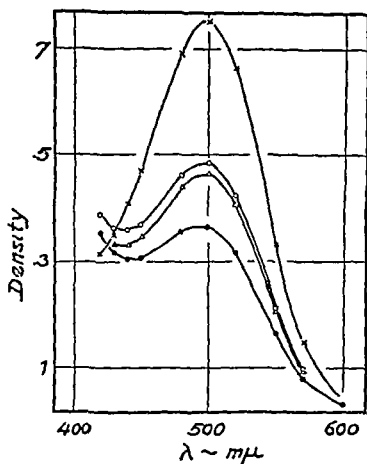


FIG 8 Effect of concentration of digitalin on keeping power of visual purple solutions at  $0^{\circ}\text{C}$ . Crosses indicate the absorption spectrum of the three solutions freshly prepared. The white circles represent the absorption spectrum after 7 weeks in the dark at  $0^{\circ}\text{C}$  of the extract made with 10 per cent digitalin, the triangles represent the 6 per cent digitalin extraction, and the black circles the 3 per cent digitalin extraction. Water extractions which contain no digitalin or other extractive usually lose all visual purple color within 48 hours in the dark at  $0^{\circ}\text{C}$ .

is known to do (Chase, 1936), or it may be that there is present a small amount of the decomposition product in the supposedly unbleached solution.

To test the influence of pH on extraction we made simultaneous preparations from equal numbers of dark adapted retinas with 2

per cent digitalin solutions buffered to pH's of 5.7, 6.6, 7.2, and 8.3 respectively

The absorption spectra are plotted in Fig. 10 as log density, and are superimposed at 500 m $\mu$ . They show clearly that the more acid the extraction, the less blue-absorbing substances are removed from the retina.

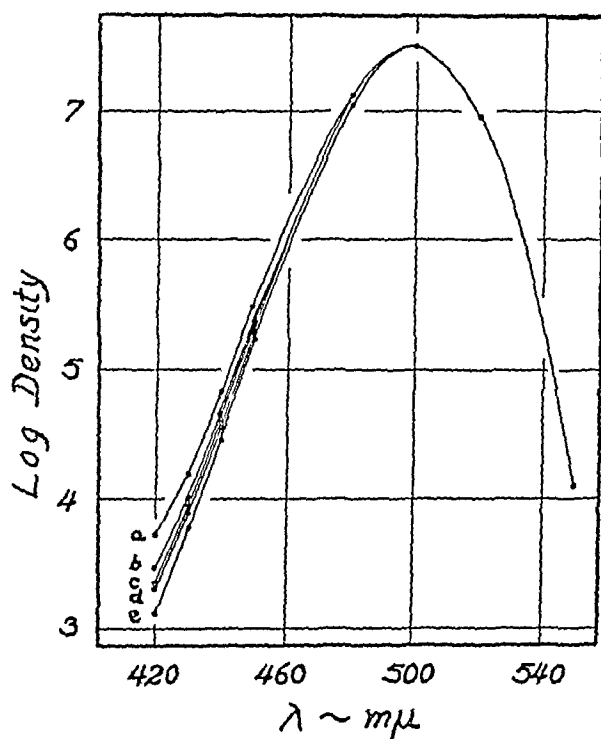


FIG. 9. Five samples of a visual purple solution buffered to different pH's after extraction. Curve *a* is pH 5.50, *b* is pH 5.76, *c* is pH 6.63, *d* is pH 7.78, and *e* is pH 10.08. Evidently a substance is present which behaves as an indicator of hydrogen ion concentration. It may be a property of the unbleached solution or a trace of decomposition product. The data are plotted as log density on an arbitrary scale after they have been made equal at 500 m $\mu$ .

(G) *Temperature*—A batch of alum-treated retinas was divided into three equal parts and extracted with 4 per cent bile salts for 10 minutes at 20°, 30°, and 40°C respectively. Fig. 11 shows that the relative amount of visual purple present is greatest in the 40° extraction and least in the 20° extraction.

In a similar experiment some retinas were extracted at 21° and some



near 0°C The low temperature extraction contained so much pigment that it required filtering and centrifuging before it could be measured, and even after this treatment most of the light absorption was clearly caused by substances other than visual purple, still probably the pigment Kuhne found that at low temperatures the pigment adheres strongly to the retina

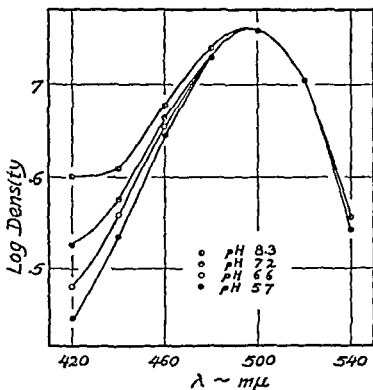


FIG 10 Absorption spectra of four simultaneous extracts made at different pH's The log density values are made equal at 500 mμ and plotted on an arbitrary log scale There is a clear effect of hydrogen ion concentration on visual purple purity in the resulting extractions Relatively more blue absorbing materials are extracted at the higher pH's

(H) *Drying*—Extractions of alum treated retinas with both 4 per cent purified bile salts solution and 2 per cent digitalin were dried by evaporation with a fan, after the absorption spectrum of a sample of each fresh solution had been measured Another sample of each was left in the undried state The dried and undried samples were kept at 0° in the dark, the former in cans containing CaCl<sub>2</sub>,

When redissolved after 25 days, the dried bile salts extraction showed considerable loss of visual purple with no gain in purity Since bile salts are hygroscopic, it is unlikely that the preparation

remained dry during the 25 day period, even though  $\text{CaCl}_2$  was present. The control sample showed about the same loss as the dried sample over this period, and a considerable precipitation of grayish material appeared in the storage tube.

After redissolving the dried digitalin extract at the end of 25 days, an insoluble whitish residue remained, though all the colored material dissolved. The absorption spectrum of the redissolved preparation

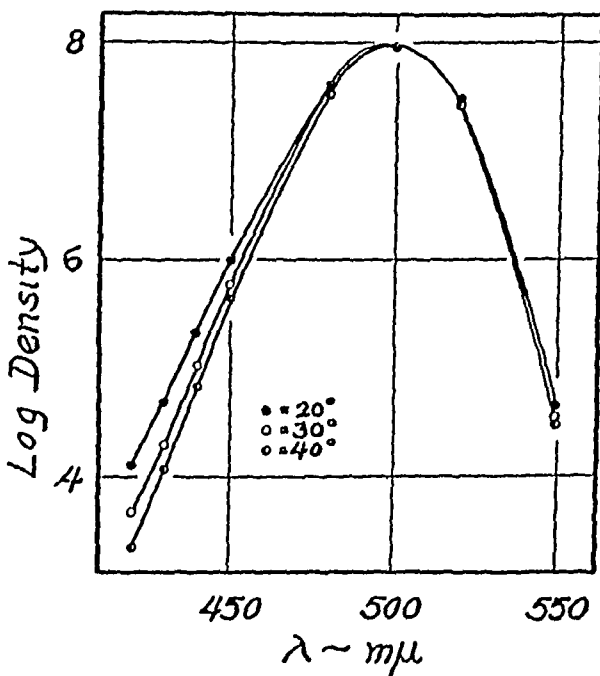


FIG. 11 The effect of temperature on otherwise identical simultaneous extractions. The log density values have been shifted on the arbitrary log scale to superimpose at 500  $m\mu$ . The purity of the visual purple color is greatest in the extraction made at the highest temperature.

was more symmetrical than that of the original fresh solution, indicating a purification of the visual purple color.

This experiment showed, incidentally, that the dried and redissolved digitalin visual purple solution kept better at room temperature over 24 hours than did the control sample. Its absorption spectrum remained almost unchanged during a day at room temperature, whereas the control sample increased in absorption in the blue and became turbid.

(I) *Other Treatments*—We confirmed Kühne's precipitation of the colored element from solutions by saturating with  $\text{MgSO}_4$ . The precipitate, after washing with saturated  $\text{MgSO}_4$ , was redissolved in water but no gain resulted in the 500/420 density ratio of the solution.

Solutions made 2 M and 4 M with respect to  $\text{NaCl}$  also showed no improvement in the 500/420 ratio, though in one case a heavy grayish precipitate occurred.

Kühne (1879) found that dialysis of a bile salts extraction against water caused precipitation of the light sensitive component. We have confirmed this both with bile salts and digitalin solutions. The precipitated reddish color has not decreased in density during 6 months in the dark at  $5^\circ\text{C}$ . It rapidly disappears upon exposure to white light but is much more stable in the dark than any solution of visual purple. We have not been able to redissolve it in water, bile salts, or digitalin solution.

#### IV

#### CONCLUSIONS

These data suggest that the classical visual purple absorption spectrum, obtained as a difference between the bleached and unbleached condition, is probably not the absorption spectrum of the visual purple molecule as a whole, but more nearly that of its light sensitive group. If this were not true, it should be possible to obtain solutions whose unbleached absorption spectra are identical with the classical difference one. Though the procedures described have improved greatly on the old absorption spectra, none of them has yielded a visual purple whose absorption spectrum is of sufficient symmetry to resemble the classical one in the violet.

The fact that the human dim visibility curve does not agree in the blue with the absorption spectrum of even our best unbleached visual purple solutions is no argument against the identity of the latter with the absorption spectrum of visual purple in solution, because the human dim visibility data may depend only on the light sensitive group in the molecule and not on the color of the protein component. However, since work now in progress shows that even our purest visual purple solutions contain proteins of larger and smaller molecular size than visual purple, final settlement of the question awaits meas-

urement of the absorption spectrum of unbleached visual purple solutions from which all proteins save visual purple have been removed

#### SUMMARY

The absorption spectra of visual purple solutions extracted by various means were measured with a sensitive photoelectric spectrophotometer and compared with the classical visual purple absorption spectrum

Hardening the retinas in alum before extraction yielded visual purple solutions of much higher light transmission in the blue and violet, probably because of the removal of light-dispersing substances

Re-extraction indicated that visual purple is more soluble in the extractive than are the other colored retinal components. However, the concentration of the extractive did not affect the color purity of the extraction but did influence the keeping power. This suggests a chemical combination between the extractive and visual purple

The pH of the extractive affected the color purity of the resulting solution. Over the pH range from 5.5 to 10.0, the visual purple color purity was greatest at the low pH. Temperature during extraction was also effective, the color purity being greater the higher the temperature, up to 40°C

Drying and subsequent re-dissolving of visual purple solutions extracted with digitalin freed the solution of some protein impurities and increased its keeping power. Dialysis against distilled water seemed to precipitate visual purple from solution irreversibly

None of the treatments described improved the symmetry of the unbleached visual purple absorption spectrum sufficiently for it to resemble the classical absorption spectrum. Therefore it is very likely that the classical absorption spectrum is that of the light-sensitive group only and that the absorption spectra of our purest unbleached visual purple solutions represent the molecule as a whole

The authors wish to state their indebtedness to Dr. Selig Hecht for many suggestions during the course of the experiments and for aid in the preparation of the manuscript. They also wish to thank Dr. Simon Shlaer for much valuable help throughout the work.

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## APPENDIX

After the completion of this paper that by R J Lythgoe on "The absorption spectra of visual purple and indicator yellow," appeared in the *Journal of Physiology*, June, 1937, number His visual purple absorption spectra are of the same degree of purity as those recorded here, a further indication that the classical visual purple absorption spectrum represents the light sensitive group in the molecule only, and that the residual blue absorption is caused by the protein nature of the molecule or by other proteins extracted with it

Our data (see Fig 9) differ from those shown in Fig 1 of Lythgoe's paper in that the symmetry of our unbleached visual purple absorption spectrum was greater at high than at low pH Our figures were the result of two independent and consistent sets of measurements

In George Wald's recent communication (1937) he criticizes any use of the classical visual purple absorption spectrum as physically meaningless because of the appearance of new pigments upon illumination of visual purple We have found, however, that above pH 9.6 these pigments absorb practically no light in the visible spectrum and consequently interfere very little with the computation of the classical visual purple absorption spectrum which we have assumed represents the photosensitive group in the visual purple molecule. The rather good constancy of the calculated values plotted in Figs 1 and 6 indicates that the measurements from which they were made could not have been greatly influenced by transient colors appearing after illumination It is true that at wave-lengths below 410 m $\mu$  complicating absorptions may occur, but they do not seem to be important over most of the visible spectrum provided the measurements are made at high pH

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# CHOLINE ESTERASE AND THE THEORY OF CHEMICAL MEDIATION OF NERVE IMPULSES

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A cogent question at the present time in regard to the theory of chemical mediation of nerve impulses is whether the choline esterase, available in the neighborhood of a nerve ending, is sufficient to destroy the acetyl choline liberated by a nerve impulse within the refractory period (1). The superior cervical ganglion of the cat is a particularly desirable tissue for enzyme studies in this connection because of the wealth of reliable data available pertaining to this organ. Recently von Brücke (2) measured the choline esterase activity of this tissue by a biological method and demonstrated a decrease in activity parallel with a degeneration of the preganglionic fibres. Other evidence of a more or less indirect nature has also been presented to indicate a relative concentration of the enzyme in the region of the nerve endings by Marnay, Nachmansohn, and coworkers (3, 4) in their studies on muscle tissue.

In the present investigation chemical measurements of the choline esterase activity of superior cervical ganglia were made under conditions carefully controlled to yield a quantitative estimation of the enzyme, and the results obtained were applied to the calculation of theoretical maximum and minimum velocities of acetyl choline hydrolysis. For these calculations it was necessary to know the value of the dissociation constant for the reaction being studied, hence a separate determination of the affinity between enzyme and substrate was performed. In the light of the calculations made, the possibility that the refractory period of the ganglion is determined primarily by the esterase action was considered. A preliminary note concerning these studies has appeared recently (5).

\* Fellow of The Rockefeller Foundation

## EXPERIMENTAL

Normal cats were killed by a blow on the head and the superior cervical ganglia were dissected out soon after. The ganglia, freed of all adhering tissue, were placed immediately in vessels fitted with stoppers and kept at  $-12^{\circ}$  until ready for enzyme study. The tissue was used within 24 hours after the animal was killed. A cylinder of tissue was removed from the frozen ganglion by means of a borer having an internal diameter of 1.71 mm, and placed on the head of a rotary freezing microtome. Sections  $25\mu$  thick were cut, and determinations of the enzyme activity in single sections were carried out by the micro procedure already described (6). The sections were each placed in a tube with 9.2 c mm 30 per cent glycerol. After 1 to 2 hours, 16 c mm of 0.1 M veronal buffer, having a pH of 8.0, and containing 0.5 per cent of freshly dissolved acetyl choline chloride, were added. Digestion was allowed to proceed for 1 hour at  $38^{\circ}$ , after which the reaction was halted by adding 50 c mm of the usual eserine-brom-thymol blue solution, and titration was performed in the manner previously described (6). The volume of each section was 0.0574 c mm, and, since the specific gravity was found to be 1.051, the weight of a section was 0.0603 mg.

The specific gravity was determined by suspending small pieces of the tissue in mixtures of bromo benzene and kerosene having different specific gravities. The specific gravity of the mixture in which the pieces remained suspended was taken as the specific gravity of the tissue.

No significant difference in activity could be observed between right and left ganglia of the same animal. The lowest average value for the enzymatic hydrolysis observed in a ganglion was equivalent to liberation of 3.03, and the highest 3.46 c mm N/20 acid in 1 hour under the conditions given. Under these conditions the degree of splitting was a linear function of time throughout the digestion period, and the substrate concentration was sufficiently high to ensure maximum velocity of hydrolysis. In a typical experiment seventeen analyses with 4 controls were conducted on one ganglion (Table I). The fairly constant enzyme activity observed throughout the ganglion would be expected from the homogeneous structure of the tissue.

A requirement of the micro method is that the digestion be carried out at a pH of 8.0. To obtain the corresponding activity at pH 7.4 reference may be made to a previous study on cat brain (6) in which the activity at pH 7.4 was found to be 0.74 times that at pH 8.0.

Since 1 c mm N/20 acid  $\approx$  9  $\gamma$  acetyl choline chloride, the quantity of this substance that can be hydrolyzed per milligram of tissue per second at pH 7.4 and  $38^{\circ}$  is then



$$\frac{3.30 \text{ c mm} \times 9 \frac{\gamma}{\text{c mm}} \times 0.74}{3600 \text{ sec} \times 0.0603 \text{ mg}} = 0.10 \frac{\gamma}{\text{sec mg}}$$

To obtain the value of the Michaelis or dissociation constant of the reaction between the enzyme and substrate the ultra micro gasometric method of Linder

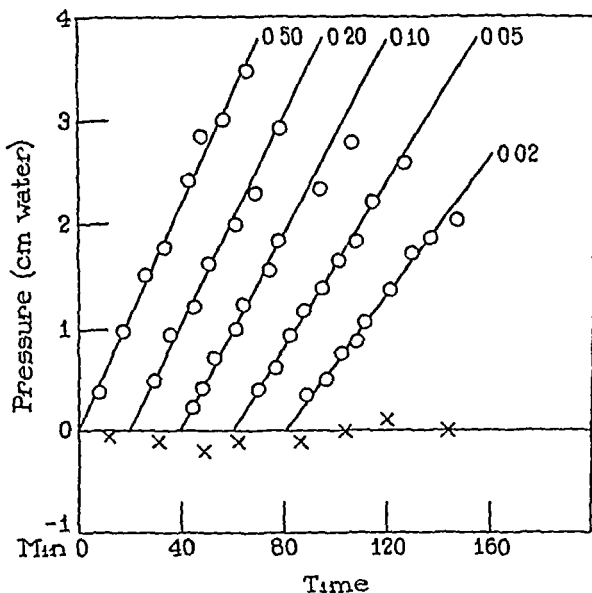


FIG 1 Initial velocities of hydrolysis of acetyl choline chloride for various substrate concentrations

Concentration of substrate (per cent) added to enzyme solutions is indicated on the lines drawn. Control experiment of barometric changes represented by points (x)

ström Lang and Glick (7) was employed since this method not only requires very little ganglion material but enables the course of the hydrolysis to be followed continuously. A ganglion weighing 15 mg. was ground with sand and Ringer's

TABLE I

*Choline Esterase Activities of Microtome Sections of Ganglion*

Microtome section No	Acid liberated in 1 hr at pH 8.0 and 38°	Acid liberated in controls
	<i>c mm N/20</i>	<i>c mm N/20</i>
1	3 63	
3	3 63	
5	3 41	
8		0 25
9	3 71	
11	3 63	
13	3 79	
14	3 43	
15	3 53	
18	3 73	
19	3 63	
21		0 41
23	3 79	
24		0 31
27	3 49	
28	3 73	
29	3 53	
30	3 75	
32	3 65	
34	3 69	
37		0 33
Average	3 63	0 33 Diff = 3 30

TABLE II

*Choline Esterase Activities As a Function of Substrate Concentration*

Concentration of acetyl choline chloride added to enzyme solution, <i>per cent</i>	0 50	0 20	0 10	0 05	0 02
Final concentration of acetyl choline, molar	0 0213	0 00852	0 00426	0 00213	0 000852
Pressure change per hr, cm water	3 30	3 10	2 85	2 40	2 00
CO <sub>2</sub> evolved per hr, corrected, <i>c mm</i> × 10 <sup>-3</sup>	33 23	29 78	28 76	22 73	15 87
Relative initial velocities of hydrolysis, <i>c mm</i> × 10 <sup>-3</sup> CO <sub>2</sub> evolved × a factor, 3.0	99 69	89 34	86 28	68 19	47 61

solution, the mixture was filtered through paper, and the residue washed on the filter with Ringer's solution until a volume of 4 ml of filtrate was obtained. 0.3 c mm of this enzyme solution was pipetted into 1.0 c mm of Ringer's solution containing the substrate (acetyl choline chloride). The pipettings were made directly into the Cartesian divers used as reaction vessels.

The 5 per cent  $\text{CO}_2$  95 per cent  $\text{N}_2$  gas mixture was passed over the substrate solution both before and after addition of the enzyme. The paraffin oil seal was placed in the neck of each diver and the measurements were conducted at  $25^\circ$  as described (7). Non-enzymatic hydrolysis of the substrate was found to be negligible under the conditions employed. Fig 1 shows the course of the reactions with various substrate concentrations. Calculations of the  $\text{CO}_2$  evolved by the reactions proceeding at the initial velocity for 1 hour were made in the

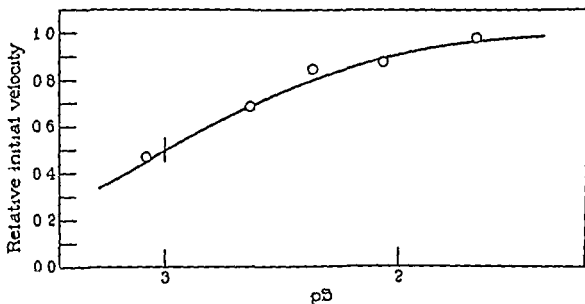


FIG 2 Activity pS relations of ganglionic choline esterase acting upon acetyl choline chloride

The curve represents the theoretical relations for a dissociation constant of 0.001, the points were derived experimentally

usual manner (7) and the results (Table II) were expressed as cubic millimeters of gas at standard conditions corrected for solubility in the aqueous phase. The points in Fig 2 were obtained from the data in Table II, and the extent of the agreement between them and the theoretical pS activity curve for a dissociation constant of 0.001 may be seen. This constant has the same value as that obtained for human serum acting upon acetyl choline chloride (8).

#### *Maximum Choline Esterase Activity*

The enzyme activity of the ganglion was shown to be equivalent to the splitting of 0.10  $\gamma$  acetyl choline chloride per second per milligram of tissue as measured under optimal conditions of substrate

concentration at a pH of 7.4 and 38°. This figure may be applied to the data of Brown and Feldberg (9) (who found in a typical experiment 22.5  $\gamma$ /gm the concentration of the ester in an unstimulated superior cervical ganglion of the cat)  $\frac{22.5 \gamma/\text{gm}}{100 \gamma/\text{gm sec}} = 0.225 \text{ sec}$  would be the time required for the enzyme to hydrolyze that amount of acetyl choline normally coexistent with it in this ganglion, provided that the enzyme and substrate were in complete combination, that is, the maximum velocity of hydrolysis were maintained. Actually the velocity falls when the substrate concentration becomes less than a certain value, hence 0.225 second represents a limiting least time which might be merely approached in actuality.

Brown and Feldberg (9) also have shown that the greatest output of acetyl choline from the ganglion perfused with eserinizied Locke's solution occurs in the first 5 minutes of preganglionic stimulation at 17 per second. In this period 0.1  $\gamma$  was liberated from a ganglion

weighing 12.9 mg. Hence  $\frac{0.1 \gamma}{12.9 \text{ mg} \times 0.10 \frac{\gamma}{\text{mg sec}}} = 0.078 \text{ sec}$  would

be the limiting least time required for the enzyme in a given weight of tissue to destroy the acetyl choline formed during the first 5 minutes of stimulation of this tissue. Furthermore  $\frac{78\sigma}{300 \times 17 \text{ stimulations}} = 0.015\sigma$  would then be the limiting least time for splitting the acetyl choline liberated by one nerve impulse. Compared to the refractory period of the ganglion which Brown (10) has found to be of the order of  $2\sigma$ , it is apparent that the enzyme need operate only with an average rate of about 0.75 per cent of its theoretical maximum velocity in order to destroy the acetyl choline liberated by a nerve impulse within the refractory period. After the first 5 minutes, the quantity of acetyl choline liberated per impulse falls until finally only about a fifth of the initial amount is set free (9), under these conditions the enzyme could hydrolyze the acetyl choline within the refractory period at about 0.15 per cent of its maximum velocity.

#### *Minimum Choline Esterase Activity*

From the foregoing it would appear that the enzyme present is sufficient to destroy the acetyl choline liberated by a nerve impulse

within the refractory period. However, it must be borne in mind that for the conditions of minimum velocity of hydrolysis, as in the case of an even distribution of enzyme and substrate throughout the tissue, the reaction velocity would be very far indeed from the maximum one, because of the low substrate concentration and affinity for the enzyme. Since  $\frac{dS}{dt} = V_{\max} \frac{S}{K + S}$  where  $S$  represents the substrate concentration,  $t$  the time,  $V_{\max}$  the maximum velocity of hydrolysis, and  $K$ , the Michaelis constant for the affinity between enzyme and substrate, it follows that the time for splitting 99 per cent of the substrate is given by  $t = \frac{1}{V_{\max}} \int_{s_1}^{s_2} \frac{K + S}{S} dS$  where  $s_2$  is the original substrate concentration and  $s_1$  is 1 per cent of  $s_2$ . From this equation we have

$$t = \frac{1}{V_{\max}} \left( K \ln \frac{S_2}{S_1} + S_2 - S_1 \right) = \frac{4.6 K + 0.99 S_2}{V_{\max}}$$

The value of  $S_2$  or the concentration of acetyl choline developed by one nerve impulse becomes

$$\frac{0.1\gamma}{12.9 \text{ mg} \times 5100 \text{ stimulations}} = 1.5 \times 10^{-6} \frac{\gamma}{\text{mg}}$$

or

$$\frac{1.5 \times 10^{-6}}{181} = 8.3 \times 10^{-9} \text{ molar}$$

Since it was shown earlier in this paper that the  $K$ , for the case in question is 0.001, as determined by the procedure described, it follows that

$$t = \frac{(4.6 \times 0.001) + \left( 0.99 \times 8.3 \times 10^{-9} - \frac{9 \text{ mols}}{\text{liter}} \right)}{\frac{0.1 \text{ mols}}{181 \text{ liter sec}}} = 8.3 \text{ sec.}$$

In order that this time be reduced to the refractory period of  $2\sigma$ , it would be necessary for the enzyme and substrate to be concentrated within a small portion of the total ganglionic volume, such as at the nerve endings. Evidence for a localization of this type has already been mentioned (1-4).

The calculations given serve to show the great divergence between the times for splitting under minimum and maximum velocities, and

hence the requirement of a state of localization of enzyme and substrate within the ganglion cell if the nerve-liberated acetyl choline is to be destroyed within the brief span of the refractory period

#### SUMMARY

The maximum choline esterase activity of the superior cervical ganglion of the cat was measured and found to be, on the average, equivalent to the splitting of 0.10  $\gamma$  of acetyl choline chloride per second per milligram of fresh tissue at a pH of 7.4 and 38°. The least possible time required for destruction of the ester liberated by one nerve impulse was calculated to be 0.015  $\sigma$ .

The dissociation constant of the reaction between the enzyme and acetyl choline chloride was determined, and a value of 0.001 was obtained.

From the value of the dissociation constant, the time for hydrolysis at the minimum rate was calculated to be about 8 seconds.

It was shown that a localization of enzyme and substrate within the ganglion cell would have to exist in order that enzymatic destruction of acetyl choline liberated by nerve impulses occur within the span of the refractory period.

The author wishes to express his appreciation of the interest with which Prof. S. P. L. Sørensen has followed this work, his gratitude to Dr. K. Linderstrøm-Lang for his invaluable suggestions and discussion, and his thanks to Dr. W. L. Doyle and K. Mogensen for providing the ganglia used. It is also a pleasure to acknowledge the stimulating suggestions afforded by Dr. G. L. Brown of the National Institute of Medical Research, London.

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## THE EFFECT OF AUXINS ON PROTOPLASMIC STREAMING II

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In connection with the analysis of the action of auxins on plant tissues, we have made a study of the relation between auxin and the streaming of protoplasm. It was shown in the preceding paper (Thimann and Sweeney, 1937) that very dilute solutions of indole 3 acetic acid increase the rate of streaming in the epidermal cells of the *Avena* coleoptile. The effect is proportional within certain limits to the concentration of indole 3 acetic acid used, and it is probably connected with the fact that it is an auxin, since two other substances having auxin activity behave in the same way, while substances which are not auxins produce no increase in streaming rate. However, the effect of these auxins on streaming was found to differ from their effect on growth in two important particulars.

- 1 While low concentrations of auxin accelerate the streaming, higher concentrations retard it. Nevertheless, these higher concentrations are those which give the greatest growth of immersed sections of coleoptiles.

- 2 The acceleration (or retardation) is transient, being over within 20-30 minutes, while the effect on growth lasts for 24 hours or more.

The present paper is mainly concerned with the reasons for these two differences. It is believed that the explanation which our experiments yield throws additional light on the mechanism of auxin action.

The methods used were the same as those previously described. However 'Segerhavre' (Victory) oats were used in the majority of the experiments in place of the Cornellian strain previously used. In several series of observations the two types have been found strictly comparable, and in fact curve I of Fig. 11 represents means of values obtained with both strains.

The influences mainly studied have been those of carbohydrates, oxygen tension, and age

## I

*The Rôle of Carbohydrates*

As mentioned above, the effect of auxins on streaming is characteristically of short duration, *i.e.* the rate of streaming returns to normal within about 30 minutes after the first application of auxin, although fresh auxin is being continually applied

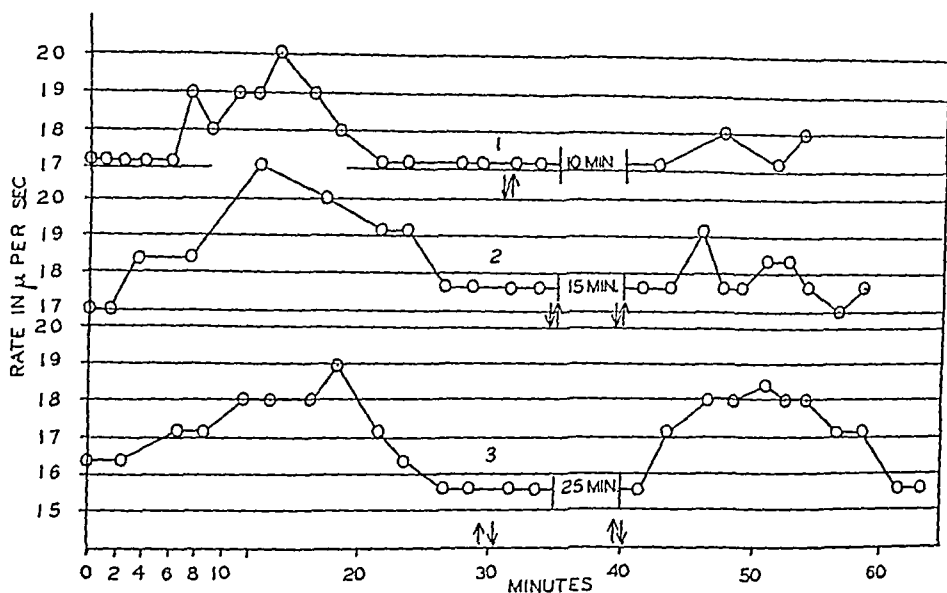


FIG 1 The recovery of the ability to react to auxin All auxin solutions 0.01 mg per liter Curve 1, auxin replaced at arrows by fresh auxin solution Curve 2, auxin replaced at first pair of arrows by water and 15 minutes later by fresh auxin Curve 3, auxin replaced at first pair of arrows by water and 30 minutes later by fresh auxin 5 cm coleoptiles aerated water

If, however, the readings in auxin solution, 0.01 mg per liter, are continued for an hour or more, the rate of streaming in the epidermal cells of the coleoptiles shows a second slight upward trend which suggests a return of the auxin effect This indication led to the following experiments About 30 minutes after the beginning of application of auxin, or as soon as the rate had returned to normal, the auxin solution was removed and replaced by fresh, aerated water The streaming then continued at the normal rate After a definite interval of



ne, auxin was again introduced. If this interval were about 30 minutes or more, at 24°C, an acceleration of streaming was again observed, approximately equal in amount to that obtained during the first auxin treatment (Fig 1). The coleoptiles have thus completely recovered their ability to react. This recovery must be due to the generation of the factor which has been exhausted, the subsequent experiments show that this factor is sugar.

If the streaming rate be allowed to return to normal, and, instead of a pure auxin solution, a solution of the same concentration of auxin in

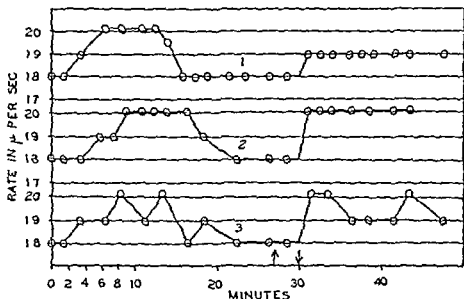


FIG 2 The effect of different concentrations of fructose on recovery. Plants treated with auxin 0.01 mg per liter until first arrow. Solution then withdrawn. At second arrow solution replaced by auxin in fructose. Curve 1 fructose 0.2 per cent, curve 2 fructose 1 per cent, curve 3 fructose 2 per cent. 5 cm. coleoptiles in distilled water.

When a concentrated sugar solution be now applied, an immediate rise in streaming rate is obtained. The period of about 30 minutes required for recovery thus completely disappears if sugar is present.

Fig 2 shows the effect of treatment with different concentrations of fructose, and indicates that 1 per cent is sufficient to cause the complete return of auxin sensitivity.

Fig 3 compares the activity of a number of different carbohydrates in a 1 per cent solution. It will be seen that fructose causes an immediate rise, sucrose and maltose a slower rise, which, however, reaches the same final rate. Curve E shows that soluble starch acts in the

same way which, in view of its large molecular size and consequent slow rate of entry, is rather surprising. It is noticeable, however, that its effect resembles that of maltose, and may perhaps be ascribed to the maltose present in such heat-treated starch. The effect of

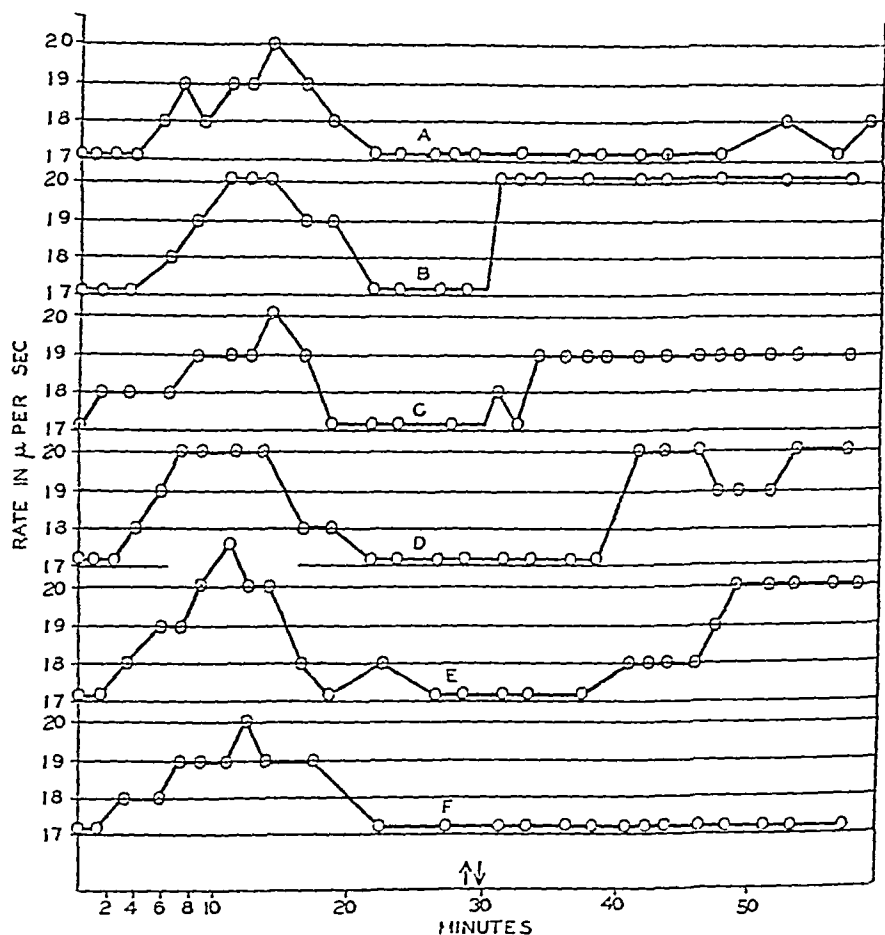


FIG 3 Recovery in solutions of different carbohydrates. Experiments as Fig 2. At second arrow, solutions replaced by (curve A) fresh plain auxin, (curve B) auxin in 1 per cent fructose, (curve C) auxin in 1 per cent sucrose, (curve D) auxin in 1 per cent maltose, (curve E) auxin in 1 per cent soluble starch, (Curve F) auxin in 0.33 per cent NaCl. 5 cm coleoptiles aerated water.

the sugars is not simply osmotic, for curve F shows that the same molar concentration of NaCl has no such effect.

Further, so long as the sugar-auxin solution is supplied, the increased rate is maintained. This is made clearer by the experiments

of Fig 4, in which the auxin is applied in sugar solution from the start. Under these conditions, the streaming rate rises in the usual way to its maximum value and remains there. The increased rate was found to remain constant for at least 2 hours. Fig 4 also shows that the application of fructose alone to the coleoptile is without any effect. The fructose curve shows a higher initial streaming rate because carried out later in the year (see section IV).

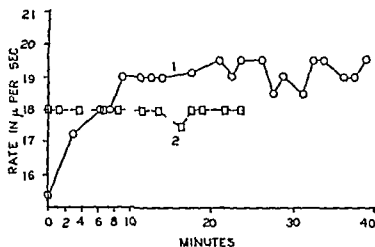


FIG 4 Curve 1, auxin 0.01 mg per liter in 1 per cent fructose (mean of two experiments) Curve 2 1 per cent fructose alone 5 cm coleoptiles aerated water the rate at time 0 is the rate in water

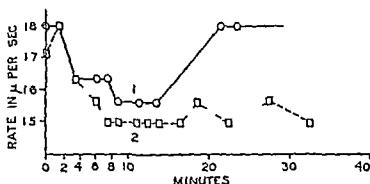


FIG 5 Curve 1, auxin 1 mg per liter, curve 2 auxin 1 mg per liter in 1 per cent fructose 5 cm coleoptiles aerated water

This action of sugars in maintaining the increased rate of streaming makes it at once possible to correlate the effect of auxin on streaming with its effect on growth. In the growth experiments, which are always conducted over periods of more than 30 minutes, the coleoptile is able to regenerate its carbohydrate supply (from storage products) continuously, and thus to respond to auxin over long periods.

The carbohydrate reserve is probably starch, since it has been shown that statolith starch is usually present in *Avena* and other coleoptiles even when grown in the dark (von Guttenberg, 1912, Zollikofer, 1918). The stored carbohydrate could not be sucrose, since sucrose itself gives a very *rapid* effect (see Fig 3), while the coleoptile evidently requires 15 to 30 minutes to obtain a fresh supply of active carbohydrate from the stored form.

Since concentrations of indole-3-acetic acid greater than about 0.5 mg per liter cause a decrease in streaming rate, it was of interest to determine whether, in the presence of sugar, this decrease was also maintained. As Fig 5 shows, it is maintained. Thus auxin concentrations which, alone, cause a temporary decrease in rate, cause a lasting decrease if they are applied together with fructose.

## II

### *The Rôle of Oxygen*

The experiments described above, as well as those in the preceding paper, were all carried out with coleoptiles 5 cm long. When the experiments were extended to younger coleoptiles, it was found that in these the streaming rate does not remain constant in aerated water, but falls steadily just as does that of 5 cm coleoptiles in unaerated water. If, however, oxygenated water was used, the rate remained constant. The 3 cm coleoptiles therefore have a greater oxygen requirement. Now Bonner (1934) has shown that the rate of oxygen uptake of 5 cm coleoptiles is not a great deal lower than that of 3 cm coleoptiles. Thus, at 77 hours, the  $Q_{O_2}$  (i.e. cubic millimeters  $O_2$  consumed per hour) was 0.31 per mm coleoptile length, while at 100 hours, the corresponding figure was 0.21. Hence, it is possible that the oxygen requirement of 5 cm coleoptiles is only *just* being satisfied by aerated water. The experiments were therefore repeated in oxygenated water, which was prepared by bubbling oxygen from a tank through redistilled water cooled in ice. Using auxin solutions made up in this water, the decrease in rate of streaming caused by higher concentration is not obtained. Instead, there is an *increase* in rate about equal to that given by a concentration of 0.01 mg per liter. Fig 6 compares the "total effect" curves for coleoptiles in aerated and in oxygenated solutions. The total effect, as defined in

the preceding paper (Thimann and Sweeney, 1937) is a measure both of the extent of the acceleration and of its duration, and is obtained by measuring the area between the curve for auxin treated plants and the curve for controls. The curve for aerated solutions is similar to that given in the preceding paper, but the values obtained in July and August are somewhat lower than those previously obtained in the winter (see section IV). Higher concentrations than were previously used were included in this series.

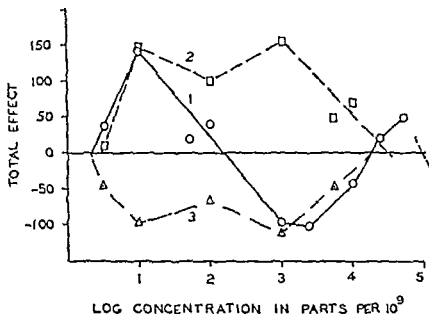


FIG 6 The total effect of auxin on streaming. Curve 1, auxin in aerated water, curve 2, auxin in oxygenated water, curve 3, auxin in aerated water containing 1 mg per liter DNP. Each point the mean of 1-12 determinations. Observations made July-August, 1937, 5 cm coleoptiles.

It follows from Fig 6 that the decrease in rate of streaming in auxin concentrations between 0.5 and 10 mg per liter in aerated water is due to a deficiency in oxygen. No deficiency of oxygen occurs when the coleoptiles are not treated with auxin, since the controls maintain a constant rate in aerated water or inactive solutions. Hence, the higher concentrations of auxin must increase the oxygen consumption of the coleoptile. This deduction was confirmed by artificially increasing the oxygen deficiency still more.

Numerous workers have shown that 2,4-dinitrophenol (DNP) increases the oxygen consumption both of animal and of plant cells (see, for instance, Plantefol, 1932). *Avena* coleoptile sections were

therefore placed in solutions of DNP and their streaming rates followed. Fig 7 shows that with 5 cm coleoptiles, the streaming rate, which remains constant in aerated water, decreases steadily in aerated DNP solutions. The fall is extremely rapid at 100 mg per liter (curve 1), moderately rapid at 10 mg per liter (curve 2), and is not observable at 1 mg per liter (curve 3). Immediately upon removing the DNP solution, the streaming rate returned to normal. Hence the action of DNP is in no way permanent, and evidently consists simply of an increase in the oxygen consumption. This point is borne

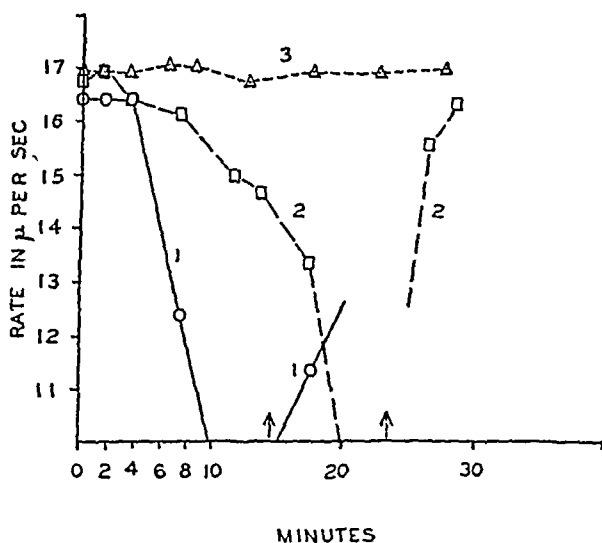


FIG 7 The effect of DNP on the streaming rate. Solutions withdrawn at arrows, subsequent readings being in air. Curve 1, 100 mg per liter withdrawn at first arrow, curve 2, 10 mg per liter, withdrawn at second arrow, curve 3, 1 mg per liter. Each curve the mean of 2 experiments. 5 cm coleoptiles aerated water.

out by the experiments on 3 cm coleoptiles described in the next section.

Since a concentration of 1 mg per liter DNP just causes no observable decrease in streaming, it presumably increases the oxygen consumption only to the point of balance, at which any further increase in the oxygen consumption will immediately become observable as an oxygen deficiency. Auxin solutions were therefore made up in aerated water containing DNP at this concentration. It was at once found that auxin concentrations which in pure water would

cause an increase in streaming, cause a decrease when in 1 mg per liter DNP. Fig 8 shows the action of 0.01 mg indole 3 acetic acid alone and in presence of DNP. It is clear that 1 mg DNP per liter reverses the sign of the auxin effect, while the lower DNP concentration gives an intermediate result.

The total effect of auxin on streaming over a series of auxin concentrations in the presence of 1 mg per liter DNP is plotted in Fig 6, curve 3. All the auxin concentrations studied *decreased* the streaming rate when in DNP. It follows, therefore, from this and the preceding data, that all auxin concentrations, except those above 10 mg

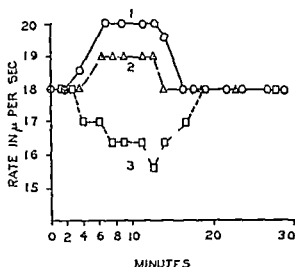


FIG 8 Modification of the effect of auxin by DNP. Curve 1, auxin 0.01 mg per liter alone; curve 2, auxin 0.01 mg per liter plus DNP 0.2 mg per liter; curve 3, auxin 0.01 mg per liter plus DNP 1 mg per liter. 5 cm coleoptiles aerated water.

per liter, increase the oxygen consumption of the coleoptile tissue. This provides further proof that the decrease of streaming observed above in aerated water is due to oxygen deficiency.

We have attempted to bring about the opposite effect of DNP, *i.e.* to reduce the oxygen consumption of the tissues in general, by treatment with dilute cyanide solutions. However, cyanide, at  $1 \cdot 10^{-4}$  and  $5 \cdot 10^{-4}$  molar, reduced the streaming rate. The effect of cyanide is immediate and is therefore probably a direct effect on the streaming process itself. Simply draining off the cyanide does not immediately restore the initial rate, as in the case of oxygen deficiency, but 5–10 minutes elapse before the normal rate is again ap-

proached That cyanide should exert a direct effect on streaming seems significant in view of Bonner's finding (1936) that cyanide inhibits growth of the *Avena* coleoptile

Since, then, the retarding effect of high auxin concentrations is due to oxygen deficiency, the curves for growth and streaming may now be reconsidered It is clear that they are approximately parallel The effect of auxin increases steadily not only up to 0.01 mg per liter, but up to about 2 mg per liter, this latter increase being shown not by increased rate of streaming, but by *an increased oxygen consumption*

The effects of still higher concentrations of auxin will be considered below (see Discussion, sections *E*, *F*, and *H*)

Two small points remain to be mentioned Since oxygenation of the water has so marked an effect, it seemed possible that still higher concentrations of oxygen might further increase the streaming rate Accordingly, observations were made on sections supported at the surface of an auxin solution in such a way that only the under half of the cylinder was immersed, and the cells under observation were actually in air The concentration of oxygen in air is about eight times as high as in oxygenated water However, the total effect of a given auxin concentration was no greater under these conditions than in oxygenated water under the coverslip Further, the maximum streaming rate, which is reached 7–15 minutes after the application of auxin, is about  $20\mu$  per second, *ice*, is also no greater Hence, the maximum rate is not limited by oxygen

Secondly, the maximum rate is also not limited by sugar This follows from numerous measurements with auxin plus fructose in oxygenated water, which gave no consistently higher rates than those without the sugar At the maximum rate the streaming is therefore limited by some other factor

In experiments with sugar, since the acceleration or retardation is maintained for a long time, it is difficult to assign values to the total effect We have, however, plotted the mean maximum rates reached against auxin concentration, and in this way have obtained curves very similar to the total effect curves Using this procedure, it was found that the presence or absence of sugar does not affect the range of auxin concentrations over which acceleration, or retardation, is



produced. The results with DNP also furnish maximum rate curves similar to those of the total effect.

### III

#### *The Behavior of Younger Coleoptiles*

(A) *The Dependence of the Streaming Rate on Oxygen*—As mentioned above, the response of 3 cm coleoptiles (77–78 hours old) was studied for comparison. Since the plants have a much higher oxygen requirement, their streaming rate does not remain constant in aerated

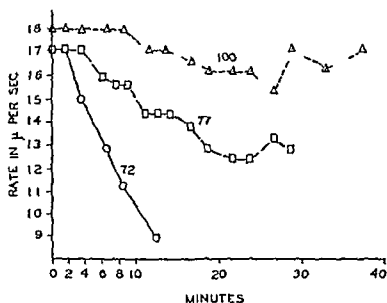


FIG 9 Streaming rate in limited quantities of water. Coleoptiles 72, 77, and 100 hours old respectively, each in 0.4 cc. aerated water not renewed.

water, but does so in oxygenated water. It has already been shown by Bottelier (1935) that the younger the coleoptile, the more readily can its streaming rate become limited by oxygen, and this, as mentioned in section II, is borne out by Bonner's measurements of respiration rates. In our experiments, the solution is ordinarily kept continuously changed by diffusion so that the oxygen deficiency may be partially made up. In order to determine the relative extents of oxygen deficiency at different ages, readings were taken on sections placed in just as much aerated water as the space under the cover slip would hold, *i.e.*, 0.4 cc. This water was not changed. Fig 9 shows that the rate in coleoptiles 72 hours old falls off with great rapidity, in 100 hour coleoptiles very slowly, and in 77 hour coleop

tiles (3 cm long) at an intermediate rate. This decrease in rate is solely due to oxygen deficiency, for as soon as the solution is removed, the rate returns to normal. This return is so rapid that intermediate streaming rates cannot be measured. Fig 10 illustrates a series of experiments in which the solutions were changed in this way. At the first arrow, when the streaming rate had fallen to an approximately constant value, the water was removed, giving instantaneous recovery of the normal rate. After 5 minutes, fresh water (curve 1), auxin solution (0.05 mg per liter) in the same water (curve 2), and auxin solution (1.0 mg per liter) in the same water (curve 3) were

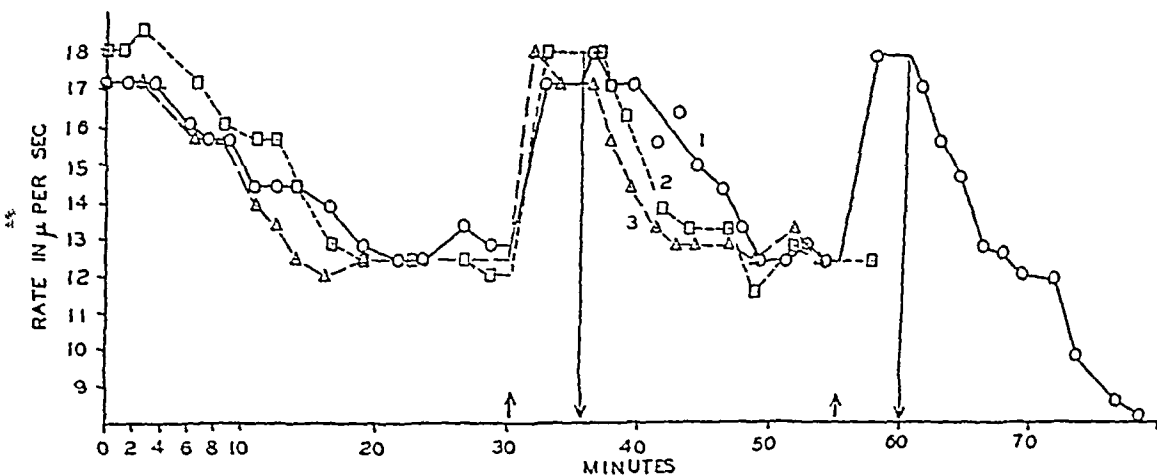


FIG 10 A comparison of the effects of auxin and DNP in increasing the oxygen deficiency of young coleoptiles. Explanation in text. Aerated water, 0.4 cc not renewed. 3 cm coleoptiles, 77 hours old.

applied. It will be seen that the decrease in rate begins in the auxin solution after 2 minutes, but in the water only after 5 minutes. The slope for the coleoptiles in auxin is also steeper than in water. The effect of the auxin in increasing the oxygen consumption is thus clearly seen. When a constant low streaming rate had been reached, the solution was again removed from one coleoptile, and, after recovery, dinitrophenol (2 mg per liter) in the same water was applied. Here the decrease in rate begins before any reading can be taken, the slope is still steeper than before, and the rate reaches a very low value. Hence the effect of auxin is in this respect exactly comparable to that of DNP, only not so great. In the light of the dis-

cussion below, this is probably because DNP increases the whole of the respiration, while auxin increases only part of it

(B) *The Effect of Auxin on Streaming*—Using oxygenated water, therefore, the effect of auxin on 3 cm coleoptiles was determined, the total effect being evaluated as previously described. The duration of the accelerating effect and the maximum value obtained were the same as in 5 cm coleoptiles

Curve 1 of Fig 11 shows that the active auxin concentrations are very much lower than those for 5 cm coleoptiles, the lowest effective

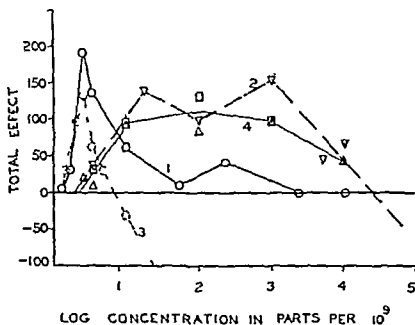


FIG 11 The total effect of auxin on streaming. Curve 1, oxygenated water 3 cm coleoptiles. Curve 2, oxygenated water 5 cm coleoptiles (cf curve 2 of Fig 6). Curve 3, aerated water 3 cm coleoptiles. Curve 4, oxygenated water 3 cm coleoptiles decapitated 90 minutes (triangles) and 180 minutes (squares) before start of experiment. Auxin 1 mg per liter = 3 on abscissae

concentration being about 0.0014 mg per liter, as against 0.0033 for the latter. The entire curve is thus displaced to the left. The right hand arm of the curve differs from that of the 5 cm coleoptiles, however, in that the higher auxin concentrations do not retard the streaming below that of coleoptiles in water (compare curve 1 of Fig 11 with curve 1 of Fig 6). The reason for this lies in the fact that even at high auxin concentrations, these coleoptiles, in oxygenated water, are only partially oxygen deficient, i.e., their "oxygen surplus" is greater than that of 5 cm coleoptiles in aerated water. This is

shown by a comparison with the curves for the same material in the same range of concentrations, but made in aerated water (curve 3 of Fig 11)<sup>1</sup> Here the left hand arm is the same as in oxygenated water, *i.e.* the material is sensitive to lower concentrations of auxin than are the older coleoptiles The right hand arm, however, shows close similarity to the curve for 5 cm coleoptiles in aerated water (curve 1 of Fig 6), *i.e.*, higher auxin concentrations cause a retardation of rate When the oxygen supply is reduced, therefore, application of auxin brings about a definite oxygen deficiency

If it were possible to determine the effect of higher concentrations of auxin on streaming rates in still greater oxygen tensions, the total effect curve would doubtless closely parallel that for 5 cm coleoptiles This is shown by the one determination we have been able to make on 3 cm coleoptile sections supported at the surface of the solution in air At 0.05 mg per liter, the value obtained (mean of two experiments) was 124, which, as will be seen, falls exactly on the curve for 5 cm coleoptiles in oxygenated water (curve 2 of Fig 11)

(C) *The Effect of Decapitation*—A remarkable feature of the response of 3 cm coleoptiles is the great effect of decapitation Curve 4 of Fig 11 shows that if the coleoptiles are decapitated 90 minutes before the start of measurements, the total effect curve is quite different from that of intact 3 cm coleoptiles, the range of effective concentrations is now equal to that of 5 cm coleoptiles In this curve, the values for plants decapitated 90 and 180 minutes are plotted separately, but since they agree closely, a mean curve is drawn through them all Thus, depriving the coleoptiles of their normal stream of auxin and other exudates from the tip for 1½ hours brings them approximately into the physiological condition of older coleoptiles, which have been naturally deprived of these secretions by the physiological senescence of the tip

(D) *Histidine A Comparison with Fitting's Experiments*—In a series of experiments, Fitting (1929, 1936) showed that, when *Valisneria* leaves are kept in pure water, their streaming ceases If leaf extract, or any one of a number of pure substances, be now applied, the streaming recommences, one of the most active of such substances

<sup>1</sup> Since under these conditions no constant rate is obtained in the controls, the points on this curve are only approximate

is *L*-histidine. In our experiments, previously reported (1937), it was shown that histidine has, however, no effect on the normal streaming of 5 cm coleoptiles. The experiments on 3 cm coleoptiles, however, suggest an explanation of the rôle of histidine, because when the streaming rate under oxygen deficient conditions is at a low value, it could be brought back to normal, either by increasing the oxygen supply, or by supplying histidine. Thus histidine presumably acts by decreasing the respiration. In the experiments of Fig 12, coleoptiles 72 hours old were allowed to remain in 0.4 cc

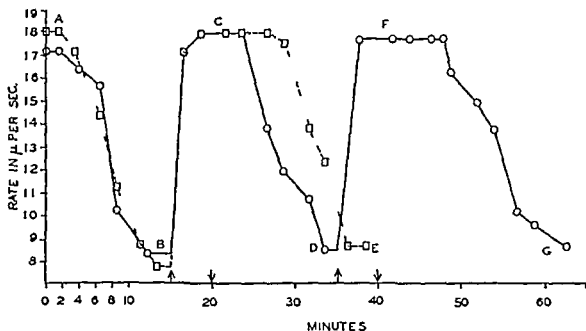


FIG 12 Effect of histidine on the streaming rate in limited quantities of water. Coleoptiles 72 hours old, in 0.4 cc aerated water, replaced at second arrow by 0.4 cc aerated histidine  $6 \times 10^{-7}M$  (CE) or by 0.4 cc aerated water (CD), again replaced at fourth arrow by 0.4 cc aerated histidine  $6 \times 10^{-7}M$  (FG). In histidine, oxygen deficiency appears only after 8 minutes; in water after 2-4 minutes.

aerated water, and the rate at which they became oxygen deficient was determined (cf also Fig 9). The curves show how closely the decrease in rate is repeated from experiment to experiment. The water was then withdrawn and after recovery in air, an aerated solution of pure *L*-histidine 0.1 mg per liter, or  $6 \times 10^{-7}M$ , was applied to one of the coleoptiles, and fresh aerated water to the other; the rate at which the coleoptiles became oxygen deficient was again determined. The solutions were then once more withdrawn and, after recovery in air, histidine solution was applied to the coleoptile

which had previously been only in water. Thus the effect of histidine is given by comparing FG with CD and AB, or CE with CD (Fig 12). It is clear that in the presence even of this low concentration of histidine—which is about equal to the threshold concentration active in Fitting's experiments—the rate of onset of oxygen deficiency is much less than normal and hence histidine must reduce the respiration of the coleoptiles.<sup>2</sup> In other experiments, it was found that when the streaming rate had fallen to a low value, the application of histidine, at the same concentration, increased the rate almost to normal. Clearly if the stoppage of streaming in immersed *Vallisneria* leaves be also due to oxygen deficiency, then the streaming

TABLE I  
*Initial Rates of Streaming in Pure Water*

Month	Nov, 1936	Dec, 1936	Jan, 1937	Feb, 1937	Mar, 1937	
Lowest	9.5	10.6	11.7	10.8	12.9	
Highest	18.0	15.7	14.4	16.4	17.1	
Mean	12.4	12.4	12.9	13.3	14.7	
P. E. of mean	0.44	0.41	0.15	0.19	0.19	
Month	April, 1937	May, 1937	June 1937	July, 1937	Aug, 1937	Sept, 1937
Lowest	14.4	14.4	14.4	14.4	16.4	16.4
Highest	16.4	17.1	16.4	17.1	18.0	18.0
Mean	15.3	16.0	15.3	15.5	17.6	17.4
P. E. of mean	0.21	0.14	0.58	0.16	0.12	

would be restarted by reducing the respiration. Whether all the substances which start streaming in *Vallisneria* act by reducing the respiration rate is of course not demonstrated, but it seems highly probable that this is the effect of histidine.

Recently Fitting (1937) has shown that also auxin restarts the streaming in *Vallisneria*, but only in concentrations above 0.0001M, i.e., 18 mg per liter. If restarting is due to a decrease of respiration, then auxin would not be expected to bring it about except at very high concentrations. Reference to curve 2 of Fig 6 shows that 18

<sup>2</sup> In unpublished experiments Bonner (1934) found that histidine  $3 \times 10^{-3}$  molal reduced respiration of coleoptile sections by 25 per cent.

mg per liter falls well on the downward slope of the curve, where auxin appears to be decreasing the respiration. This satisfactorily supports the above explanation.

#### IV

#### *The Variation of Streaming Rate with Time of Year*

During the course of these experiments, it was found that the streaming rate of controls in pure water became steadily faster. In November 1936, the mean was  $12.4\mu$  per second and in August, 1937  $17.6\mu$  per second. Table I summarizes the data from all our observations. With the exception of June, 1937, the means are derived from at least twenty observations each. If the results are classified by time of day they show no consistent variation, a fact which was also noted by Bottcher (1934). Coincident with the higher values from May to August, the effect of added auxin was found to be less, both at low and at high concentrations. The total effect curve (Fig. 6) determined in July and August is noticeably flatter than that determined in the winter and given as Fig. 4 of the preceding paper (Thimann and Sweeney 1937).

#### DISCUSSION

The experiments raise a number of new points, not all of which can be immediately explained. Some call, however, for special consideration.

In the experiments with carbohydrates, one of the most surprising conclusions is that simple sugars evidently enter the cell with extreme rapidity. The effect of fructose is observed within a minute of application. This stands in clear contrast with classical experiments on permeability, in which the sugars are always the slowest substances to enter. Whether this contrast is due to the use of mature, non growing cells in permeability experiments, as opposed to the young, growing cells of coleoptiles cannot be said. The sugar must actually penetrate the cell in these experiments, since the inactivity of NaCl shows that the effect cannot be a simple osmotic one.<sup>3</sup>

All the results agree in showing that auxin not only accelerates streaming, but also increases the respiration of the coleoptile. Yet neither Bonner (1936) nor van Hulssen (1936) could find any increase of respiration in sections of *Avena* coleoptile on treatment with auxin.

<sup>3</sup> Permeability experiments, however, depend upon the entry of sugar into the vacuole, our data only indicate its entry into the protoplasm. Nevertheless this may be physiologically more significant than entry into the vacuole.

solutions This discrepancy is easier to understand if we consider the following three facts First, the increase of respiration caused by auxin may not be very large It is great enough to cause coleoptiles which had an adequate oxygen supply, when in aerated water, to become oxygen deficient when auxin is added It is not, however, great enough to induce an oxygen deficiency when the 5 cm coleoptiles are in oxygenated water It is possible, indeed, that the decrease in streaming rate provides a very delicate indicator of increased respiration Second, the increase in the absence of added fructose is transient, and is mostly over within 20–30 minutes Since the rate rises to a peak in 10–15 minutes with an immediate subsequent fall, the *mean* increase of rate over a 30 minute period is only 14–20 per cent of the rate in the controls (see Fig 2 of Thimann and Sweeney, 1937) Third, concentrations of auxin up to 0.01 mg per liter (equal to 1 on the log abscissa) evidently cause little increase of respiration, a marked increase, as measured by development of a marked oxygen deficiency, is caused only by concentrations above 0.1 mg per liter This concentration is about 10 units per cubic centimeter Neither Bonner nor van Hulssen used auxin concentrations higher than this

While it is hoped to carry out respiration measurements at a later date, we feel at present that the failure to detect an increase of respiration on treating coleoptile sections with pure auxins is sufficiently explained by these three considerations

The only fact which does not fit in with this interpretation is the failure of auxin concentrations above 0.05 mg per liter to increase the oxygen deficiency (as measured by retardation of streaming) of coleoptiles in dinitrophenol Up to about 0.05 mg auxin per liter the oxygen deficiency in DNP increases regularly with increasing auxin concentration (curve 3 of Fig 6), but beyond this point the curve in DNP (1 mg per liter) approaches the curve in plain aerated water, meeting it at 1 mg auxin per liter At this concentration, however, there are equal numbers of molecules of auxin and DNP present per unit volume (molecular weight of indole-acetic acid = 175, molecular weight of DNP = 184), and this suggests that some interaction is taking place between auxin and DNP, whereby the action of the DNP is inhibited That such an interaction occurs is supported by observations on more dilute DNP solutions In con-



centrations of 0.2 mg DNP per liter, which, as Fig. 8 shows, still exert considerable influence on the respiration, the total effect curve in DNP meets the curve in plain aerated water at an auxin concentration of 0.2 mg per liter. That is, while 1 mg DNP is rendered ineffective by 1 mg auxin, if only 0.2 mg DNP is present it is rendered ineffective by 0.2 mg auxin. At auxin concentrations above this point of ineffectiveness, the streaming response is not altered by the presence of DNP (Fig. 6, curve 3).

There is also some chemical evidence that compounds might be formed between DNP and indole 3 acetic acid (see the discussion in Sidgwick, 1937).

We can now return to consideration of the fundamental problem—the mechanism of the action of auxin. It was shown above that while low concentrations of auxin accelerate streaming, higher concentrations in presence of a limited oxygen supply actually retard it. This may be interpreted as follows—

The respiration of the coleoptile comprises the oxidation of several substrates, of which sugar is doubtless one of the most important. One of these oxidation processes controls the rate of protoplasmic streaming, and is itself evidently accelerated by auxin. Its oxygen consumption must be only a small fraction of the whole. If now we make one assumption, namely that auxin also accelerates another oxidation process, which does not control the rate of streaming, the observed facts can be satisfactorily explained. We have the following system

- I. Sugar + O<sub>2</sub>  $\xrightarrow{\text{auxin sensitive}}$  protoplasm streaming and growth
- II. Sugar + O<sub>2</sub>  $\xrightarrow{\text{auxin sensitive}}$  no effect on streaming
- III. Other substrates + O<sub>2</sub>  $\xrightarrow{\text{auxin insensitive}}$  no effect on streaming

The discussion will be made clearer by reference to the lettered sections of the diagrammatic Fig. 13.

*Section A*—Low concentrations of auxin accelerate reactions I and II, the acceleration of I causing an acceleration of streaming. It is important that these concentrations (0.0033 to 0.01 mg per liter for 5 cm coleoptiles) are also the lowest which cause an increase in growth rate. The 3 cm coleoptiles, which give a growth response to

somewhat lower auxin concentrations, correspondingly give a streaming response down to 0.0014 mg per liter

*Section B*—Higher auxin concentrations bring about a greater acceleration of reaction I, but since both I and II consume oxygen, there will be a competition for oxygen. The result will be that the effect of auxin on I cannot be fully exerted—we get a smaller acceleration of streaming rate

*Section C*—Still higher auxin concentrations accelerate reaction II to such an extent that oxygen is actually withdrawn from I, which is therefore retarded

*Section D*—This retardation does not, of course, occur in oxygenated water, instead, the acceleration of I is maintained over a considerable range of auxin concentrations, being limited here by some other fac-

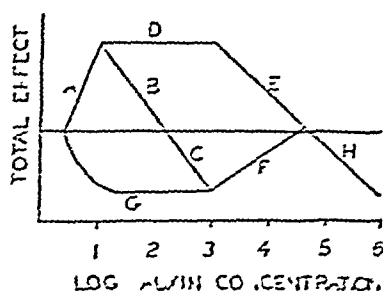


FIG. 13 Total effect curves diagrammatic. ABCFH in aerated water, ADEH in oxygenated water, GF in aerated water plus DNP 1 mg per liter. 5 cm coleoptiles. Auxin 1 mg per liter = 3 on abscissae

tor, such as perhaps the amount of the enzyme for this reaction. The observations at the end of section 2 make it clear the limiting factor cannot be oxygen or sugar.

The fact that the total effect does not increase with increasing auxin concentration above 0.01 mg per liter, i.e. that section D is parallel to the axis, agrees with some measurements of the growth of coleoptile sections floating on auxin solutions for 1 hour. In this short period the agreement between streaming and growth should be at its best. Correspondingly the measurements show about the same increment for all auxin solutions from 0.01 to 10 mg per liter.

*Section G*—In the presence of dinitrophenol the effect of auxin is the opposite of that in section A. Here reaction III, which comprises other oxidative systems of the cell, is accelerated and conse-

quently the tissues are at the verge of oxygen deficiency, any acceleration of I and II leads to an increased demand for oxygen and I is retarded on account of it. This explanation, as also that of sections B and C, depends on the "affinity" of reaction II for oxygen being greater than that of reaction I, so that II can withdraw oxygen from I. This may be due to a difference between the enzymes of I and II, a larger amount of its enzyme present, or other reasons. Although II thus differs from I in its oxygen affinity, its auxin sensitivity is evidently the same as that of I.

The fact that section G meets section C at about 1 mg auxin per liter has been discussed above. By contrast with DNP, the action of cyanide is evidently exerted directly on reaction I, although it may involve reactions II and III as well.

*Section E*—At high auxin concentrations, above 1 mg per liter, the streaming in oxygenated water is accelerated less. These, then, are the concentrations at which the effect of auxin on reactions I and II begins to be reduced, i.e., the "supramaximal" zone.

*Section F*—Correspondingly, in aerated water, since II is less accelerated the oxygen demand is less and hence I is retarded less, i.e., the "aerated" and "oxygenated" curves both approach zero.

*Section H*—At auxin concentrations above about 50 mg per liter the system is evidently damaged, for the streaming rate falls irreversibly. This final lowering is not reversed by the presence of oxygen and is evidently due to some toxic effect of high auxin concentrations. It is noteworthy that these concentrations are those which cause actual shrinkage of the coleoptile. This may be seen by comparing Fig. 6 with the growth curve given as Fig. 5 in Thimann and Sweeney (1937).

In the range of sections E and F, it is possible that a discrepancy exists between the curves for growth and for streaming. Auxin concentrations of 20 and 100 mg per liter cause a considerable increase in growth during the 1st hour, but have only little effect on streaming. Since the amount of growth in the period covered by the streaming measurements is very small, however, it is hard to obtain satisfactory data for it. Curvature measurements, by the agar technique, certainly show decreased angles in this range. Further experiments will be necessary to decide this point.

In conclusion, the close parallel between the effect of auxin on streaming (in presence of sufficient oxygen) and the effect of auxin on growth—a parallel which the present experiments have greatly extended—indicates that the reactions giving rise to accelerated streaming and to growth are either the same or are mutually interdependent. While streaming may go on without growth, as in resting cells, old coleoptiles, or other non-growing tissues, it is probable that growth cannot go on without streaming. Hence in the chain of auxin-induced reactions, the effect on streaming doubtless *precedes* that on growth. This is also supported by the extremely rapid onset of the effect on streaming.

The experiments and the theory outlined above provide an explanation for the close relationship between growth and respiration elucidated by Bonner (1936). They fit in well with the findings of Schneider (1938) that the action of auxin on the coleoptile is largely dependent on the presence of sugar, and that under suitable conditions growth may be limited either by auxin or by sugar. They suggest also that the effect of light on streaming, studied by Botteher (1934) may be exerted through an effect of light on auxin relations. They give a tentative explanation for the remarkable results of Fitting. Finally, they provide a satisfactory basis for the known fact that auxin brings about a number of apparently independent responses in the cell, since an acceleration of protoplasmic movement, through accelerating the movement of nutrients or other substances in the protoplasm, might bring countless other effects in its train.

#### SUMMARY

1 A further study has been made of the effect of indole-3-acetic acid (auxin) on protoplasmic streaming in the epidermal cells of the *Avena* coleoptile.

2 The transient nature of the effect of auxin, both in accelerating and retarding streaming, is due to the temporary exhaustion of carbohydrate from the tissues. In presence of 1 per cent fructose or some other sugars the acceleration or retardation of streaming by auxin is not transient, but is maintained for at least 2 hours.

3 The retardation of streaming brought about by concentrations of auxin above 0.5 mg per liter is due to oxygen deficiency. This has been confirmed in several ways.

4 It follows that the effect of auxin is to increase the respiration of the coleoptile tissue

5 Younger coleoptiles, 3 cm long, are sensitive to lower concentrations of auxin than those 5 cm long, and more readily exhibit oxygen deficiency as a result of the action of auxin. However, after decapitation their response to auxin more closely resembles that of 5 cm coleoptiles

6 The retardation of streaming in such coleoptiles, resulting from oxygen deficiency, is delayed by very dilute solutions of histidine. On this basis an explanation is suggested for the results of Fitting on streaming in *Vallisneria* leaves

7 The mean rate of streaming in control untreated coleoptiles in pure water varies with the time of year, but not with the time of day

8 The results support the view that auxin accelerates an oxygen consuming process which controls the rate of protoplasmic streaming, and that the latter controls growth. The substrate for this process is probably sugar

9 It is suggested that auxin also accelerates another oxygen consuming process, which may withdraw oxygen from the process which controls streaming rate and hence cause retardation of the latter

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# CRITICAL INTENSITY AND FLASH DURATION FOR RESPONSE TO FLICKER WITH ANAX LARVAE

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## I

To test the generality of the phenomena observed when the flicker response curve is obtained with differing proportions of light time to dark time in a flash cycle experiments were made with the larvae of *Anax junius* (dragonfly) in addition to those, already discussed, with the sunfish (Crozier, Wolf, and Zerrahn-Wolf, 1937-38d) The flicker curve ( $F - \log I_m$ ) for *Anax* (1936-37a, b) is of special interest in that, while it is not complicated by the presence of two sorts of peripheral sensory elements, the convex surface of the eye mechanically introduces a distortion of the curve due to the comparative inefficiency of ommatidia at the margin of the eye (1937-38b) As a consequence, the probability integral which efficiently describes the flicker data in the case of vertebrates (1936-37c, 1937-38a, c, d, Crozier, 1937) is widely departed from by the *Anax* measurements at lower intensities The discrepancy is explained by the results of blocking out parts of the eye (1937-38b) It was predicted that the departure from the probability integral would be reduced by increasing the proportion of light time to dark time  $t_L/t_D$  in a flicker cycle

The procedure and apparatus have been described in the preceding papers The *Anax* larvae used were of the same lot as those in our examination of the rôle of areas of eyes (1937-38b) Check experiments with  $t_L/t_D = 1$ , at 21.5°, gave close agreements in the values of  $I_m$  with those previously found under these conditions The variation in  $I_1$  agreed with that observed in other individuals of this group (1937-38b), but for both was lower than that in an earlier series (1936-37b) This is presumed due to differences in the groups of individuals

The measurements are summarized in Table I and Fig. 1

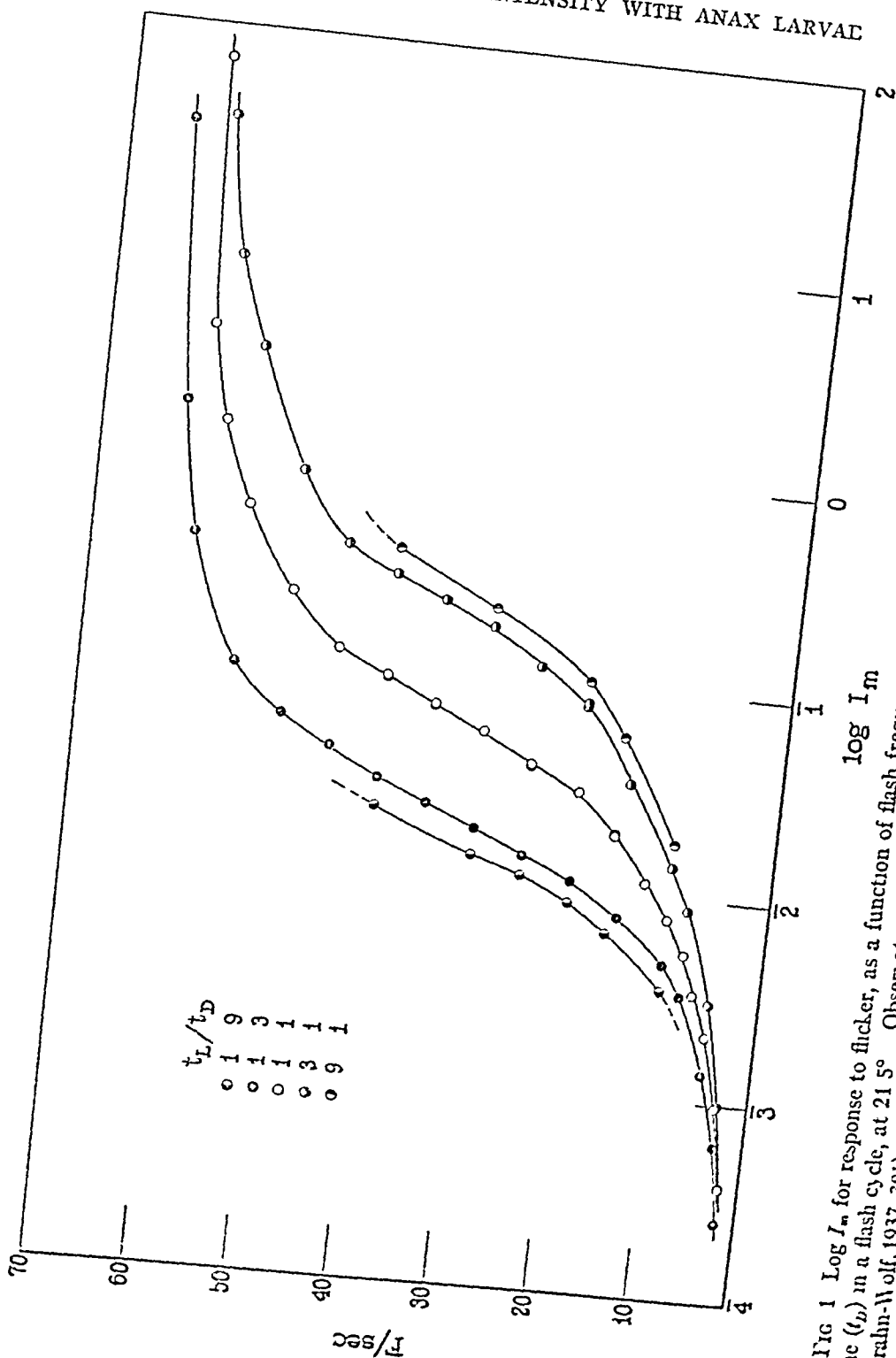


FIG. 1.  $\log I_m$  for response to flicker, as a function of flash frequency  $F$ , with different proportions of light time ( $t_L$ ) to dark time ( $t_D$ ) in a flash cycle, at 21.5°. Observations on larvae of *Anax* (data in Table I and, for  $t_L/t_D = 1$ , from Crozier, Wolf, and Zerrahn-Wolf, 1937-38b). (Technical difficulties make it impossible to obtain complete curves for  $t_L/t_D = 1/9$  and 9, consequently no values of  $I_{max}$  for these are estimated (cf. Figs. 2 and 3).)



## II

Precisely as with the sunfish (1937-38d), increase in the light time fraction moves the flicker curve (Fig 1) toward higher intensities and a

TABLE I

Mean critical illumination  $I_m$  in a flash and  $P E_{I_1}$  (millilamberts) as a function of flash frequency  $F$  per sec, for different percentages of light time ( $t_L$ ) in a flash cycle, at 21.5°, for larvae of *Anax junius*  $N =$  ten individuals,  $n =$  three observations on each, at every point (Data on  $t_L = 50$  per cent are given in Crozier, Wolf and Zerrahn Wolf 1937-38b)

$t_L$ per cent	10		25		75		90	
$F/sec$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$	$\log I_m$	$\log P E_{I_1}$
2			$\bar{4}$ 4151	$\bar{6}$ 9262	$\bar{4}$ 5924	$\bar{6}$ 8295		
3			$\bar{4}$ 7950	$\bar{5}$ 2716	$\bar{4}$ 9997	$\bar{5}$ 3510		
5			$\bar{3}$ 1480	$\bar{5}$ 5227	$\bar{3}$ 4982	$\bar{5}$ 9384		
8			$\bar{3}$ 5233	$\bar{4}$ 1065	$\bar{3}$ 9451	$\bar{4}$ 2405		
10	$\bar{3}$ 5469	$\bar{5}$ 8278	$\bar{3}$ 6729	$\bar{4}$ 2054	$\bar{2}$ 1526	$\bar{4}$ 7837	$\bar{2}$ 2693	$\bar{4}$ 8005
15			$\bar{3}$ 8887	$\bar{4}$ 3059	$\bar{2}$ 5462	$\bar{3}$ 1232		
16	$\bar{3}$ 8064	$\bar{4}$ 1433					$\bar{2}$ 7697	$\bar{4}$ 9593
20	$\bar{3}$ 9454	$\bar{4}$ 0409	$\bar{2}$ 0500	$\bar{4}$ 7641	$\bar{2}$ 9219	$\bar{3}$ 6695	$\bar{1}$ 0290	$\bar{3}$ 2700
					$\bar{2}$ 9069	$\bar{3}$ 1232		
25	$\bar{2}$ 0580	$\bar{4}$ 4736	$\bar{2}$ 1535	$\bar{4}$ 3479	$\bar{1}$ 0806	$\bar{3}$ 2240		
30	$\bar{2}$ 1408	$\bar{4}$ 4398	$\bar{2}$ 2696	$\bar{3}$ 1737	$\bar{1}$ 2545	$\bar{3}$ 6675	$\bar{1}$ 3459	$\bar{3}$ 8864
35			$\bar{2}$ 3733	$\bar{4}$ 9353	$\bar{1}$ 3659	$\bar{3}$ 9155		
40	$\bar{2}$ 3434	$\bar{4}$ 7634	$\bar{2}$ 4765	$\bar{3}$ 1222	$\bar{1}$ 4695	$\bar{3}$ 8191	$\bar{1}$ 5036	$\bar{2}$ 0622
45			$\bar{2}$ 6129	$\bar{3}$ 0641	$\bar{1}$ 5938	$\bar{3}$ 9998		
50			$\bar{2}$ 7509	$\bar{3}$ 2721	$\bar{1}$ 9201	$\bar{2}$ 3090		
55			$\bar{2}$ 9779	$\bar{3}$ 4729	0 4921	$\bar{2}$ 7746		
58					0 9181	$\bar{1}$ 2591		
60			1 5801	$\bar{2}$ 1052	1 5704	0 4224		
62			0 2068	$\bar{2}$ 5982				
63			1 5324	0 4835				

lower maximum<sup>1</sup> The decrease in  $F_{max}$  is directly proportional to the increase in  $t_L/(t_L + t_D)$ , as seen in Fig 2 The values given for

<sup>1</sup> In these experiments there is no sign of the effect found when two luminous intensities are flickered (as by reflection from light and dark sectors of a rotating surface) Under such conditions (Porter 1898, etc of Piéron, 1935 Crozier Wolf and Zerrahn Wolf 1937-38d)  $F$  at fixed  $I$  in a flash passes through a maximum as  $t_L/t_D$  is increased Apparently the same condition obtains with insects for in tests involving the phototropic balancing of continuous light by a sector

$F_{max}$  are those found to give best rectilinearity of the upper parts of the curves upon a probability grid (Fig 3). Accepting this formulation as fundamentally correct, and the departures at lower intensities as caused by mechanical disadvantage of the marginal retinal elements in the reception of light, the plots in Fig 3 also show that the standard deviation of the log  $I$  distribution of  $dF_p/d \log I$  is constant, where  $F_p = 100 F/F_{max}$ . This is likewise true of the sunfish curves (1937-38*d*), and in each case is also found when temperature is varied (1936-37*b*, 1936-37*c*).

The intensity at the inflection of the ideal curve (straight lines in Fig 3) is directly proportional (Fig 8) to  $t_L/(t_L + t_D)$ , and conse-

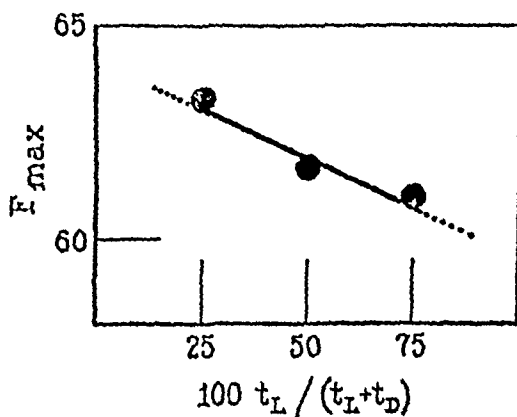


FIG 2  $F_{max}$  declines as percentage light time in the flash cycle is increased (The rate of the decrease is less than with the sunfish (1937-38*d*))

quently to  $-F_{max}$  (cf Fig 2). This again is found in the sunfish data.

The departure from a symmetrical curve for  $F$  vs  $\log I_m$  is clearly diminished as  $t_L/t_D$  is increased, as was predicted (1937-38*b*). With longer flashes, and higher mean flux of illumination at a given  $F$ , the chance of adequately involving disadvantaged ommatidia is naturally greater, although with larger  $t_L/t_D$  the frequency with which each of

beam of the same  $I$  the data of Mast and Dolley (1924) clearly show that  $I'$  at the point of equivalence of the two beams passes through a maximum. The elaboration of experiments of this type holds the possibility of really deciding whether the events determining the form of the flicker function are primarily peripheral or central.

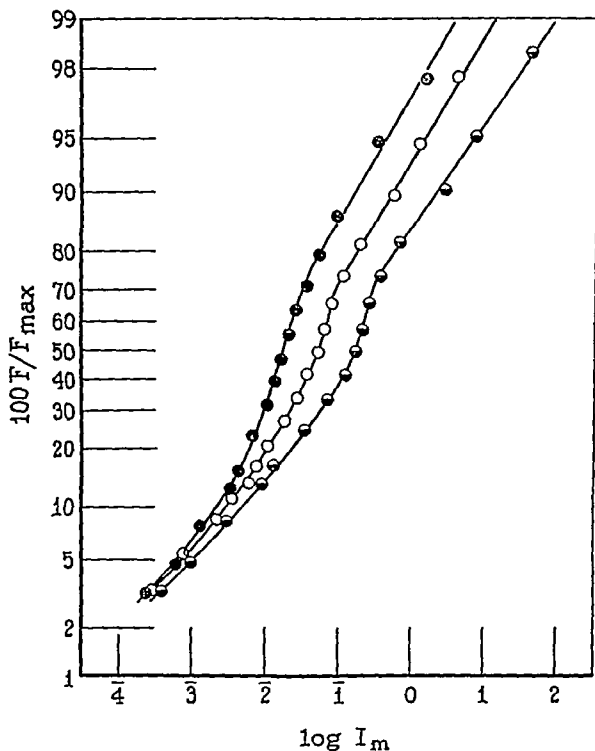


FIG 3  $\log I_m$  vs  $F$  on a probability integral grid ( $F = 100 F/F_{max}$ ) The departure from rectilinearity, over the lower range of intensities increases as the proportion of light time is made larger See text, and also Fig 4

these can be effectively excited is decreased Thus while with the sunfish the shape of each part of the flicker curve is independent of  $t_L/t_D$ , a real change in shape is produced with  $Anax$  (Fig 4) in the

lower two-thirds of the curve The maximum is not affected by this easier recruitment of peripheral units, because in any case the total of the potentially available elements is open to excitation long before  $F_{max}$  is reached

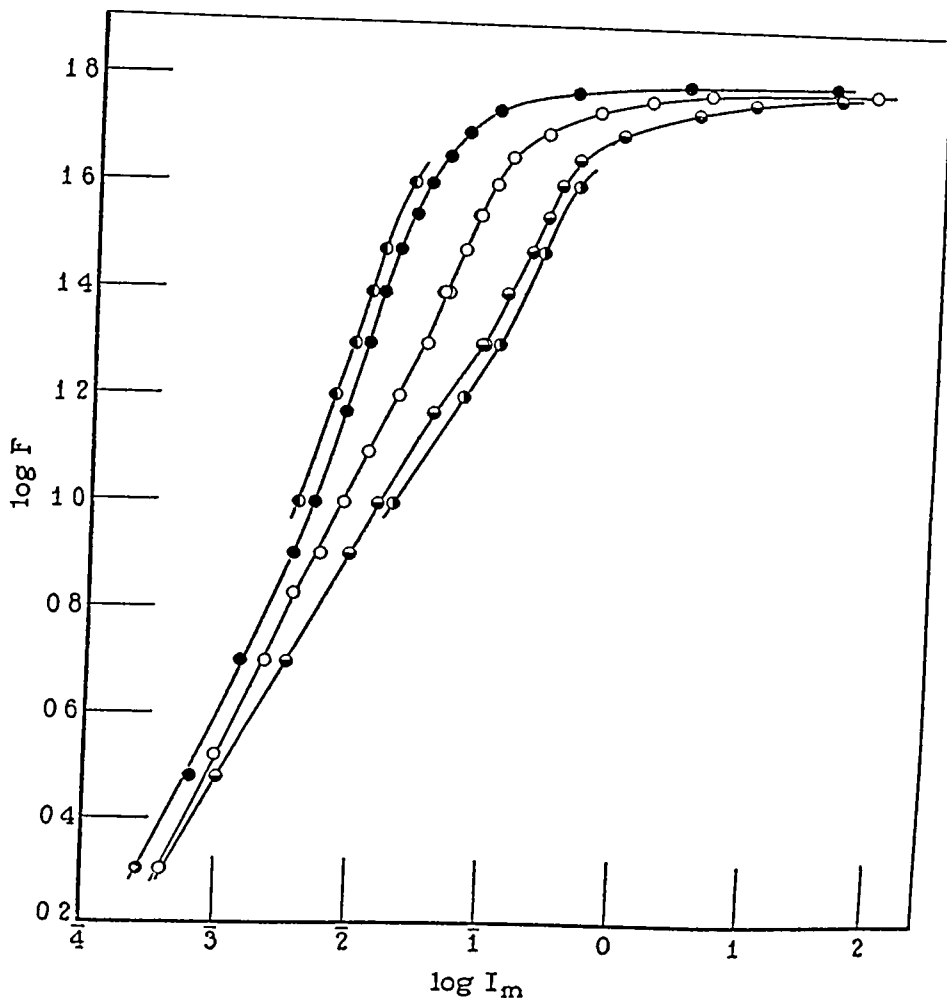


FIG 4  $\log I_m$  vs  $\log F$ , for different proportions of light time to dark time in a flash cycle, to show comparative shapes of the flicker contours

The measurements with the sunfish showed that the direct proportionality between  $I_m$  and  $PE_L$  was identical at the various  $t_L/t_D$  ratios Fig 5 demonstrates that the rule holds for *Anax* as well

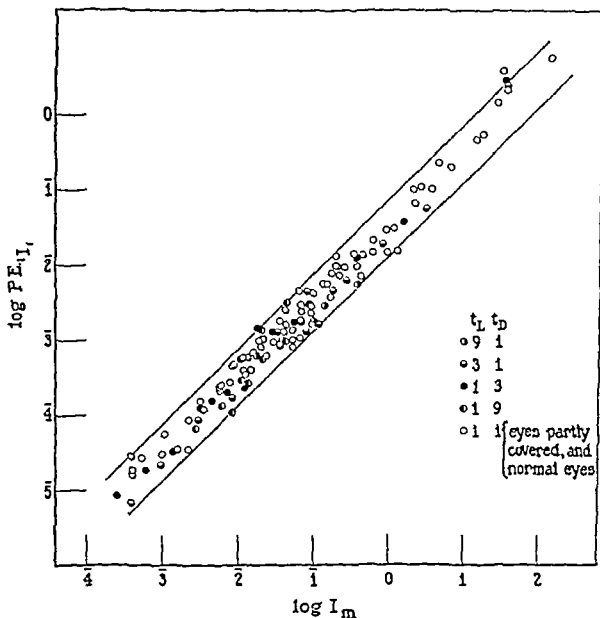


FIG 5 The variation of  $I_1$  (as  $P E_{1/I_1}$ ) and  $I_m$  are directly proportional (slope on log grid = 1) The proportionality constant is independent of visual area (open circlets data in Crozier, Wolf and Zerrahn Wolf 1937-38b) and of  $t_L/t_D$  (Table I)

### III

The basic features of the flicker curve, as described by the parameters of a probability integral, are thus identical in their behavior with respect to changes in the ratio of light time to dark time in the two very different animals, sunfish and *Anax* larva. The different behavior of these parameters with reference to temperature is also

the same in both. A similar explanation may therefore be advanced in each case (1937-38*d*). It is based merely upon the dynamical properties of the behavior which has provided the measurements. These properties are defined by their quantitative relations to the experimental variables. Their meaning apparently is that  $F$  corresponds to a summated number of elemental effects, normally distributed as a function of  $\log I$ . Each  $I_m$  is the mean intensity required to produce this summation for a particular  $F$ , its variation ( $P E_{I_1}$ ) is the intensity fluctuation corresponding to the natural fluctuation in excitability, which at each critical  $F$  is also measured by the ordinate  $dF/d \log I$  since the number of elements in effective fluctuation will be proportional to the mean number marginally excited (1937-38*a*). The natural cycle of fluctuation in the excitability of each element is to be pictured as a relaxation-oscillation (*cf* Hoagland, 1935) not affected by temperature, although the amount of excitation required for the response is increased by raising the temperature (1936-37*c*), so that  $F_{max}$  is unaffected. By shortening the time during which the light acts, in a cycle of fixed duration, it is possible for a succession of the flashes to act upon a larger number of elements before a magnitude of total summated effect is produced which will lead to forced recognition of flicker.  $F_{max}$  is then increased, in simple proportion to the percentage shortening of the light time (Fig 2), because the succession of briefer flashes and longer dark times enables more elements to be "caught" in an excitable state, just as with larger visual areas. Since on this basis the same or additional elements of the same mean excitability can contribute more frequently to the summation required for critical  $F$ ,  $\log I_m$  at the inflection point ( $= \tau'$ ) should decrease, but  $\sigma_{\log I}$  should be the same (for a homogeneous receptor field). The frequency distribution, with area at 100 per cent, is merely moved to a lower position on the  $\log I$  scale. These are the relations as found.

By comparison with the flicker curves for fishes (1937-38*a*) and for man (1937-38*c*) that for *Anax* is distinctly of an aberrant form. Such cases provide a valuable test of the general analysis, when the reasons for the unusual or less simple structure of the curve can be specified. For *Anax* this can be done, and in terms which are consistent with data on the visual performance of other arthropods.

(1937-38b) The present results are in keeping with this. They indicate clearly that if  $t_L/t_D$  were to be reduced much below  $1/9$  an even greater departure from the probability integral should be expected.

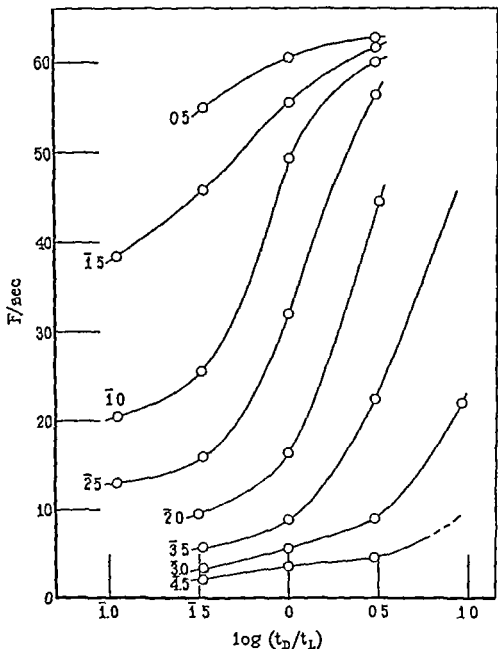


FIG. 6  $F$  vs  $\log(t_D/t_L)$ , for various values of  $\log I_m$ , as obtained from Fig. 1. For high values of  $F$  direct proportionality is more nearly approached. (This is also observed if calculations are made on the basis of  $I_e$ , the mean equivalent flux of illumination at the point of the response.)

pected in the case of an arthropod with greatly convex eyes. This condition was employed by Sälzle (1932), and his flicker curve for *Aeschna* larvae is much more asymmetrical than any of ours (i.e., the departure is greater).

This view obviously requires that changes of temperature should not modify the shape of the discrepancy in the *Anax* flicker curve, any more than they affect  $F_{max}$ . In fact on a probability grid the shape

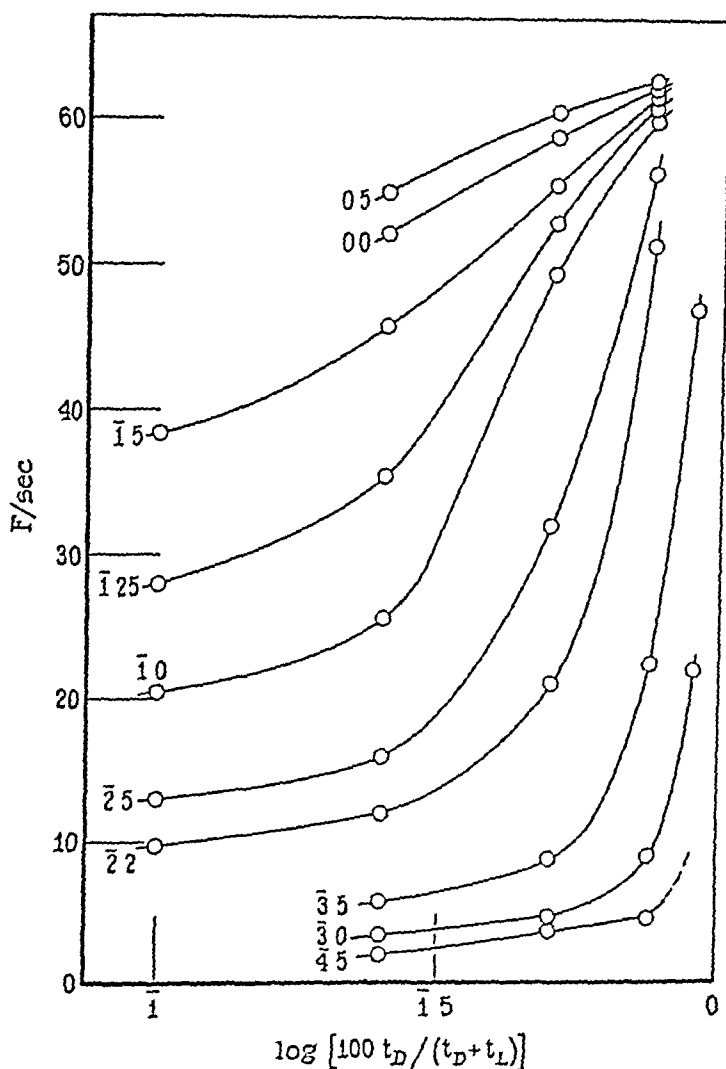


FIG. 7  $F$  at different values of  $I_0$ , as a function of the percentage dark time in the flash cycle

is identical from  $12.4^\circ$  to  $27.3^\circ$ . Increasing  $t_L/t_D$ , however, decreases the discrepancy and lowers  $F_{max}$ . It is clear that conditions immediately affecting the reception of light ( $t_L/t_D$ , area) have a recognizably different effect upon the flicker curve than those primarily modifying



the central nervous mechanism of intensive discrimination (as, temperature)

Neither the curves for sunfish nor for *Anax* obey the rules which have been suggested for somewhat similar (but less complete) experiments with man.  $F$  at fixed  $I$  is not directly proportional to  $\log(t_D/t_L)$  (Fig 6) but follows a curve which is dependent upon the magni

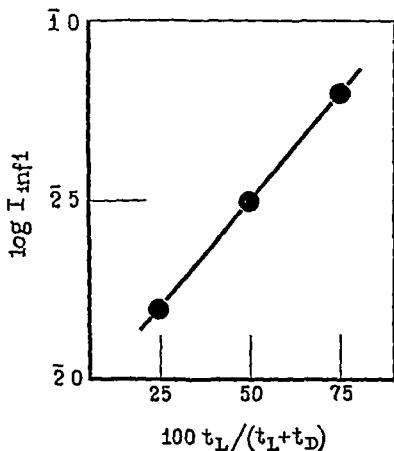


FIG 8 For *Anax* larvae, as for the sunfish (1937-38d)  $\log I$  at the inflection point of the flicker contour ( $= \tau'$  in Fig 3) is directly proportional to the percent age light time in the flash cycle

tude of  $I$ . There is no clearer case to be made out if comparisons are made on the basis of  $I_{eq}$ , the mean equivalent flux of illumination (Fig 7). Talbot's law cannot be used to bring the several curves in Fig 1 into coincidence. The simplest general fact of the situation, cleared of the structural complications, is that  $\tau'$ , the  $\log I$  at inflection of the ideal probability curve (Fig 8), varies directly as the fraction of the cycle time occupied by light.

## SUMMARY

Determinations of the flicker response curve ( $F - \log I_m$ ) with larvae of *Anax junius* (dragonfly) for various ratios  $t_L/t_D$  of light time to dark time in a flash cycle provide relations between  $t_L/t_D$  and the parameters of the probability integral fundamentally describing the  $F - \log I$  function, including the variability of  $I$ . These relations are quantitatively of the same form as those found for this function in the sunfish, and are therefore non-specific. Their meaning for the theory of reaction to visual flicker is discussed. The asymmetry of the *Anax* curve, resulting from mechanical conditions affecting the reception of light by the arthropod eye, is (as predicted) reduced by relative lengthening of the fractional light time in a cycle.

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# THE KINETICS OF EXCYSTMENT IN COLPODA DUODENARIA\*

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Protoplasmic reorganization involving a dedifferentiation to a stage of lesser structural complexity followed by a redifferentiation to a highly complex structure is a fundamental procedure which protozoa tend to follow at times of fission, conjugation, regeneration, and cystment<sup>1</sup>. The normal reproductive cycle of the small ciliate *Colpoda duodenaria*<sup>2</sup> exhibits this procedure rather markedly. In this organism the free swimming growth period is invariably followed by a resorption of cilia and other cytoplasmic structures along with the formation of a cyst within which fission is completed. Division never occurs without this dedifferentiation and cystment. By certain changes in the environment (Barker and Taylor, 1931) the normal free swimming growth period may be arrested as illustrated in Fig 1 and the protozoan induced to form a resting cyst in which division may or may not occur. Resting cysts differ from division cysts in that the redifferentiation, excystment process, does not occur unless certain constituents not found in a simple physiologically balanced salt solution are present in the surrounding medium. This ciliate is thus an excellent experimental organism since both its dedifferentiation and its redifferentiation may be induced by control of environmental conditions<sup>3</sup>.

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<sup>1</sup> For a review of this phenomenon see an earlier paper (Taylor, 1935).

<sup>2</sup> For a study of the morphology and taxonomy of this protozoan see Taylor and Furgason (1937).

<sup>3</sup> The specific compounds inducing excystment are not yet known though they are present in many complex organic extracts such as hay infusion, yeast autol

Preliminary to some experiments carried out on the effects of changes in the radiation environment on the cystment process of *Colpoda* (Taylor, Brown, and Strickland, 1936), a brief study (unpublished) was made of the influence on induced excystment of temperature, concentration of hydrogen ions, and concentration of the excyst-

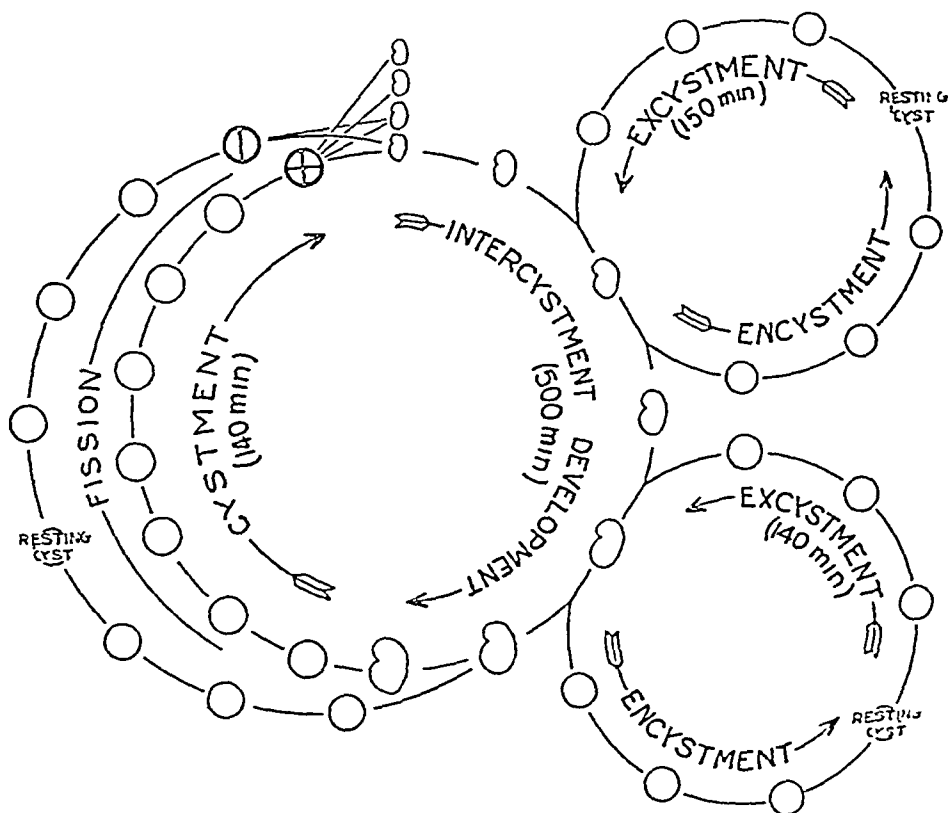


FIG 1 Reorganization cycles in *Colpoda duodenaria* and approximate duration of some stages under standardized conditions (temperature, 20°C, salts, Osterhout's balanced physiological medium, 0.012 per cent total concentration, food, adequate suspension of *Pseudomonas fluorescens*, and excystment medium, 1 per cent Difco yeast extract)

ment solution. The influence of two of these factors, namely, temperature and concentration of the excystment compounds, was

ysate, and beef extract. See Barker and Taylor (1933) and Thimann and Barker (1934) for studies seeking to ascertain the nature of these excystment inducing compounds.

also studied by Thumann and Barker (1934) as a preliminary to their investigation of the plant hormone auxin as a possible excystment inducing substance. The influence of extreme temperatures and a number of chemical reagents on *Colpoda* cysts has been studied by Taylor and Strickland (1936), Bodine (1923), Goodey (1913), and others<sup>4</sup>. However, it was deemed advisable to investigate more thoroughly these environmental factors affecting the cystment process in an attempt to gain a fuller understanding of this profound reorganization.

### *Materials and Methods*

Preparations of the resting cysts of a pure strain of *Colpoda duodenaria* aligned in grooves on Cellophane have been made as described in recent papers (Taylor and Strickland 1935, Taylor, Brown and Strickland, 1936) with some improvements, principally in the selection of organisms of a more uniform intercystment age before induction of the encystment. In the preparation used in this series of experiments a few of the organisms (about 3 per cent) divide once before completion of excystment, but no difference in excystment time between those that divide and those that do not divide has thus far been detected.

The cyst Cellophane preparation is cut in strips which are hung in continuously flowing balanced medium (Osterhout's, 0.012 per cent total salt) at 20°C. Under these conditions the same preparation yields uniform results for a period of many months. For immediate experimentation, small pieces containing approximately 150 *Colpoda* cysts are cut from the strips and placed in 0.5 cc. of balanced medium in a covered Columbia dish, which is then transferred to a second constant temperature room. In all cases at least 2 hours have been allowed for the preparation to come to temperature equilibrium and then the inorganic salt medium replaced by the excystment inducing medium. Readings of the number of unexcysted organisms are then taken regularly during the period over which organisms are emerging from their cysts.

The excystment inducing medium used throughout these experiments has been a solution of Difco bacto yeast extract (Ref. No. 283,399) in physiologically balanced salt solution (0.012 per cent total salt). Especially purified salts and triple distilled water have been used in making up this solution.

Since traces of many organic materials affect the excystment process, care has been further taken to avoid contamination. All glassware has been placed in a concentrated sulfuric nitric acid mixture and then thoroughly rinsed before each experiment. Precise control of temperature is quite essential and precautions, such as maintenance of a high relative humidity (above 85 per cent) to avoid

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<sup>4</sup> See Taylor and Strickland (1936) and Bodine (1923) for reviews of the literature.

lowering of temperature by evaporation, have been taken. All manipulations have been carried out in special constant temperature rooms which are maintained constant to within a few tenths of a degree Centigrade.

The distribution of fractional excystment as a function of the logarithm of the excystment time is found to be normal.<sup>5</sup> In practice,

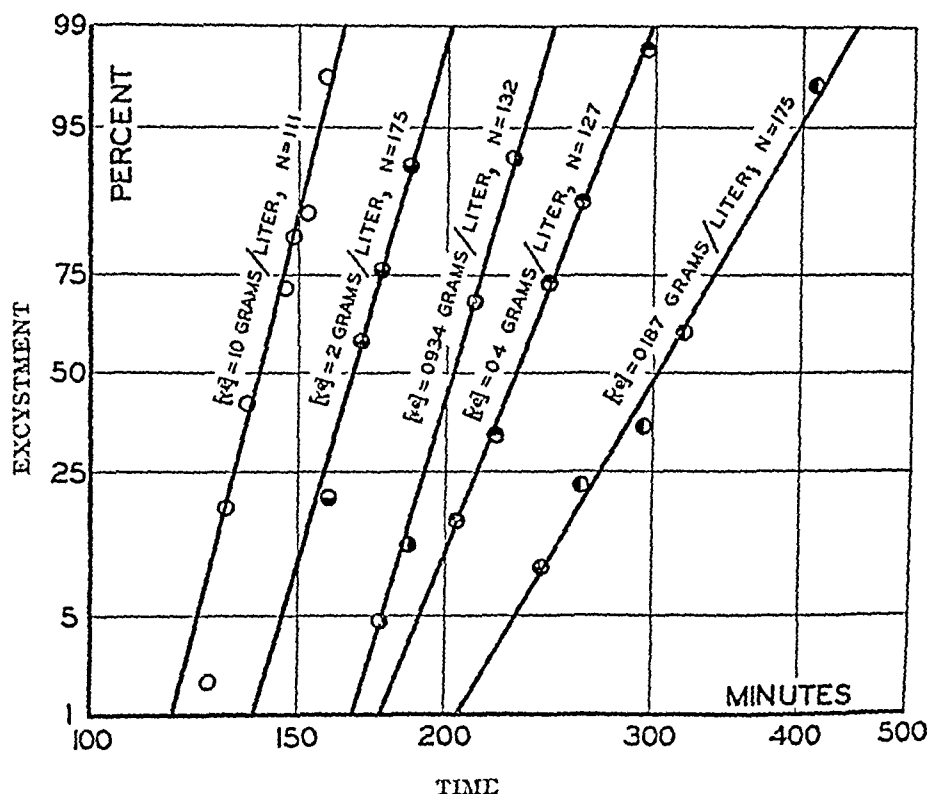


FIG. 2. A set of experimental data on the time for excystment of *Colpoda duodenaria* at 20°C.  $[ye]$ , concentration of yeast extract,  $N$ , number of organisms in test, time zero when excystment medium placed on cysts.

time has been measured in minutes, excystment in per cent, and the values of the mean log excystment time and standard deviation determined graphically from a plot of fractional excystment on a

<sup>5</sup> If  $N_t$  be the number of excysted organisms at any time  $t$ ,  $N$  the total number of organisms in the test, and  $\log t_{xx}$  be the mean log excystment time, then for  $N$  large the fractional excystment is

$$\frac{N_t}{N} = \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{\log t} e^{-\frac{1}{2\sigma^2} \log^2 \frac{t}{t_{xx}}} d \log t$$

probability scale<sup>6</sup> as a function of time on a logarithmic scale. With these scales the experimental data fall neatly on straight lines whose slope is proportional to the reciprocal of the standard deviation. Hence the excystment in any one experimental test is completely described by the values of mean log excystment time,  $\log t_{.5}$ , and the standard deviation,  $\sigma$ . A sample set of experimental data is plotted as Fig. 2 to illustrate the sort of dispersion usually found. Even though curves be anomalous with regard to others of the set, the points still fall in straight lines so that there is little difficulty in determining the values of  $\log t_{.5}$  and  $\sigma$ .

#### EXPERIMENTAL

In this investigation something of the nature of the reorganization (excystment) reactions has been determined from extensive data on the influence on excystment time of two environmental factors, *viz.*, temperature and concentration of the yeast extract of the excystment medium. These are factors which alter the rate of the reactions yet do not apparently change their nature though varied over wide ranges. The temperature range has been from 8°C to 32°C and the concentration range from 0.08 gm./liter to 22.4 gm./liter. Concentrations beyond these limits have been tested, mostly at 20°C, but found to result in only partial excystment in many instances.<sup>7</sup> The mean excystment times,  $t_{.5}$ , vary in the ranges studied from less than 80 minutes to more than 10,000 minutes.

The experimental data are summarized in Table I. The values for  $\log t_{.5}$  and  $\sigma$  are averages considering each test of equal significance regardless of the number of organisms in that test. In each test, measurements have been estimated to a hundredth of an inch on graphs of excystment on a probability scale as a function of time on a logarithmic scale. Since the logarithmic scale used is 10 inches/cycle,

<sup>6</sup> E.g., Codex Book Co., Inc., Norwood, Massachusetts. Number 3128.

<sup>7</sup> The lower limit for 100 per cent excystment of a little less than 0.1 gm./liter checks closely with the limit for dry weight concentration of hay extract to induce 100 per cent excystment found by Thumann and Barker (1934). Their tests were made at 20-23°C and the tests with yeast extract mostly at 20°C. They further showed that this limit depends only on the concentration and not on the total amount of extract present so that volume of medium used in these experiments, even at lowest concentrations, is not a critical factor.

TABLE I

*A Summary of Experimental Data*

$\theta$ , temperature, degrees Centigrade,  $[ye]$ , concentration of yeast extract, gm./liter,  $\log t_{ez}$ , mean log excystment time with  $t$  measured in minutes,  $\sigma$ , standard deviation of log excystment times for individual organisms, dimensionless,  $N$ , number of organisms, and  $n$ , number of tests.

$\theta$	$[ye]$	$\log t_{ez}$	$\sigma$	$N$	$n$	$\theta$	$[ye]$	$\log t_{ez}$	$\sigma$	$N$	$n$
32	10	1.92	0.042	448	3	15	0.187	2.66	0.106	348	2
	2	1.97	0.051	338	2		0.08	2.86	0.132	268	2
	0.4	2.10	0.068	580	3						
	0.08	2.28	0.079	57	1						
29	10	1.91	0.027	235	2	13	10	2.56	0.036	652	5
	2	1.95	0.049	390	3		4.67	2.54	0.035	188	1
	0.4	2.10	0.060	291	3		2	2.62	0.037	556	5
							0.934	2.70	0.051	253	2
26	10	1.92	0.028	376	3	12	0.4	2.82	0.062	563	5
	2	2.01	0.044	496	4		0.187	3.04	—	126	1
	0.4	2.16	0.062	617	5		10	2.72	0.055	681	4
	0.08	2.34	—	130	1		4.67	2.65	0.056	363	2
23	10	2.02	0.032	279	2	11	2	2.77	0.053	669	4
	2	2.14	0.047	189	2		0.934	2.90	0.061	261	2
	0.4	2.38	0.080	368	3		0.4	2.97	0.075	481	4
							0.187	3.21	0.075	172	1
20	10	2.15	0.028	2304	16	10	10	2.84	0.052	964	6
	4.67	2.18	0.026	665	4		4.67	2.86	0.088	303	2
	2	2.21	0.035	2315	16		2	2.95	0.080	920	6
	0.934	2.29	0.035	579	4		0.934	3.04	0.091	292	2
	0.4	2.38	0.056	2141	15		0.4	3.22	0.033	826	6
	0.187	2.52	0.079	642	4		0.187	3.46	0.078	221	1
	0.08	2.68	0.120	1096	7						
17	10	2.26	0.036	892	6	9	10	2.93	0.068	599	4
	4.67	2.31	0.033	288	2		4.67	3.07	0.083	249	2
	2	2.29	0.050	907	6		2	3.16	0.085	566	4
	0.934	2.32	0.055	261	2		0.934	3.34	0.149	370	2
	0.4	2.40	0.054	867	6		0.4	3.60	0.169	534	4
	0.187	2.52	0.127	69	1						
	0.08	2.61	0.116	315	2		10	3.23	0.073	643	4
							4.67	3.29	0.094	352	3
15	10	2.40	0.033	1127	7	8	2	3.47	0.077	684	4
	4.67	2.41	0.045	214	2		0.934	3.62	0.142	322	2
	2	2.43	0.044	1060	7						
	0.934	2.47	0.055	352	2		10	3.49	0.120	440	3
	0.4	2.55	0.075	989	7		4.67	3.64	0.151	551	3
							2	4.07	0.294	427	3



each 1/100 inch represents a change of one unit in the third figure following the decimal point. This last figure, however, is not truly significant since the standard deviation of log excystment times of individual organisms from the mean is of the order of 0.05 and the number of organisms 150 so that the mean would have a standard deviation of the order of 0.004 (equivalent to about 1 per cent on a time scale) if all conditions were the same in each test. That conditions have not been exactly the same in each test is shown by the observed deviations of the means, for the tests at 20°C and 10 gm./liter the value of  $s$  (root mean square deviation) is 0.027, for 15°C and 10 gm./liter, 0.029, for 20°C and 2 gm./liter, 0.036, for 15°C and 2 gm./liter, 0.040, for 20°C and 0.4 gm./liter, 0.066, and for 15°C and 0.4 gm./liter, 0.043. Thus the dispersion of conditions of experimentation cuts the accuracy inherent in the preparation by a factor of about ten. With this standard deviation of the order of 0.04, the probable error for  $\log t_{.2}$  from any set of tests would be approximated by  $0.03/n$  (equivalent to about  $7/n$  per cent on a time scale) where  $n$  is the number of tests.

#### *Influence of Concentration of Excystment Medium on Excystment Process*

The process of excystment in this analysis is considered to depend on a set of chemical reactions, the first of the set requiring some constituent from the medium used to induce the excystment. The time required for the complete process is then some function of the times required for the separate reactions of the set. The excystment period has been broken up into intervals depending on the nature of the change occurring during that interval.

The first splitting of the excystment period is into two intervals, one dependent on the concentration of the excystment medium and the other independent of its concentration. These two intervals have not been measured directly,<sup>8</sup> but computed from the measurements of the time for completion of the excystment process as a function of

<sup>8</sup> Some unpublished experiments by Mr. A. G. R. Strickland indicate that direct evaluation of this first interval may be made by experiments in which the cysts are washed free of the excystment medium after a limited time in it. Mr. Strickland found that if this washing is early in the excystment period no excystment takes place, whereas if late in the excystment period, all organisms complete the excystment process.

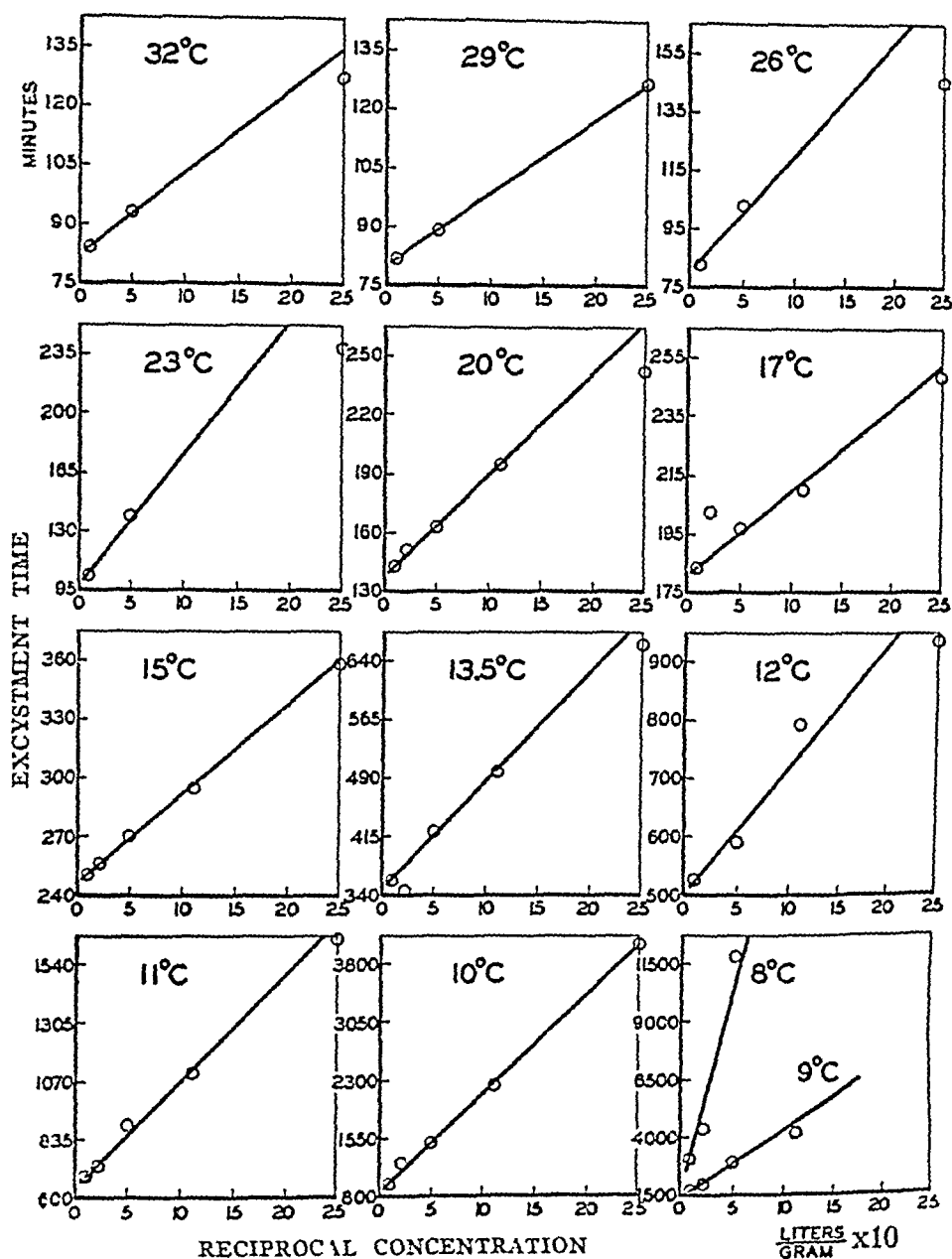


FIG 3 Excystment time for *Colpoda duodenaria* as a function of reciprocal yeast extract concentration

the concentration of the yeast extract used to induce the excystment. If the time for the interval dependent on  $[ye]$  be  $t_2$ , then it is found

that all isothermal experimental data are well fitted by setting  $t_2$  inversely proportional to  $[y c]$ . The complete excystment time,  $t_z$ , would then be the sum of  $t_2 = B/[y c]$  and a constant,  $C = t_z - t_2$ . It is seen from this relation,

$$t_z = \frac{B}{[y c]} + C \quad (1)$$

that plots of excystment time,  $t_z$ , as a function of reciprocal  $[y c]$  should be straight lines whose intercept with the time axis is the interval independent of  $[y c]$ , i.e. the constant  $C$ , and whose slope is the interval dependent on  $[y c]$  for unit concentration, i.e., the con-

TABLE II

*A Summary of Derived Data*

$\theta$ , temperature, degrees Centigrade,  $C$ , excystment period independent of concentration of excystment inducing medium, minutes,  $B$ , excystment period dependent on concentration of excystment medium for unit concentration minutes subscripts  $a$  and  $b$  reference to different methods of analysis

$\theta$	$C$	$C_a$	$\theta$	$C$	$C_b$	$\theta$	$B$	$B_a$	$\theta$	$B$	$B_b$
32	81.6	82.0	13½	348	344	32	21.2	19.0	13½	142	148
29	79.2	79.6	12	500	500	29	19.2	18.6	12	214	211
26	80.0	77.5	11	639	650	26	39.3	42.2	11	428	389
23	97.4	96.0	10	838	820	23	78.7	75.9	10	1 287	1 259
20	137.5	137.5	9	1375	1200	20	52.0	55.0	9	3 050	3 310
17	180.4	180.5	8	1500	1700	17	28.7	30.2	8	18 100	13 500
15	246	246				15	45.6	40.7			

stant  $B$ . The points for each temperature have been plotted, straight lines drawn through them, and the values of  $C$  and  $B$  determined from the time intercept and slope. Since the experimental concentrations were taken at equal intervals on a logarithmic scale, the points are very unequally spaced on a reciprocal scale and the data for the two lowest concentrations have been omitted from the graphs of Fig. 3 in order that the more accurately determined points for higher concentrations be not excessively crowded. The values of  $C$  and  $B$  determined in this way are entered in Table II as  $C_a$  and  $B_a$ .

The values of  $C$  and  $B$  have also been determined independently from the same experimental data by a second method. Equation (1)

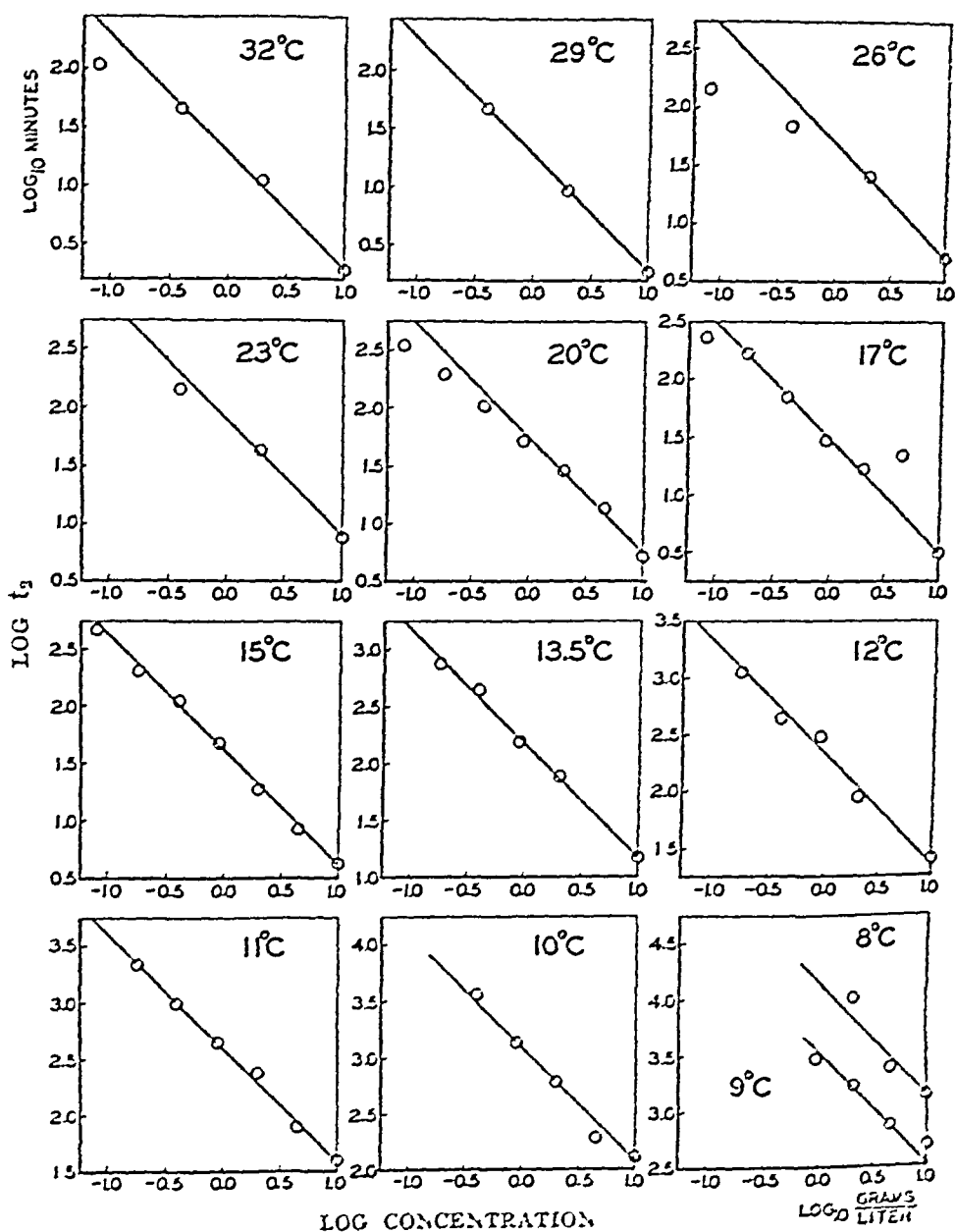


FIG. 4. Logarithm of the first excystment periods as a function of logarithm of yeast extract concentration

may be rewritten as  $(t_{sz} - C)[ye] = B$  from which it follows that  $\log(t_{sz} - C) + \log[ye] = \log B$ , and it is seen that since  $t_z = t_{sz} - C$ ,

plots of  $\log t_2$  as a function of  $\log [y e]$  are straight lines whose slopes are  $-1$ . For each set of isothermal data values of  $C$  have been determined so that the points fall as closely as possible on straight lines with slope  $-1$  when  $\log t_2$  is plotted against  $\log [y e]$ . These graphs include the experimental points for the lower concentrations and are shown in Fig. 4 and the values of  $C$  and  $B$  taken from them entered as  $C_2$  and  $B_2$  in Table II. In the subsequent analysis the average values from these two determinations have been used, though the values are so close that no marked difference would be noted if either one alone had been taken.

The relation between excystment time and yeast extract concentration, equation (1), which fits the isothermal experimental data rather well, is a relation that may be derived from simple assumptions concerning the nature and mechanism of the excystment process. One need only consider this process to depend on a compound,  $Y_1$ , of the yeast extract diffusing into the encysted organism and there reacting with a substance,  $P_1$ , of the protoplasm according to the general equation



where  $Y_1P_1$  is a complex which reorganizes to form the compound  $P_2$  which is essential to excystment.

Let the concentration of  $Y_1$  outside of the organism be  $[Y_1] = \alpha_1[y e]$  and at the place of action with  $P_1$  inside the organism be  $[Y_1]$ , then the rate of change of  $[Y_1]$  would depend on two rates, that of diffusion and that of formation of the complex  $Y_1P_1$ . This second rate is equal to  $-d[P_1]/dt$  so that the kinetic equation for this dependence would be

$$\frac{d[Y_1]}{dt} = D_1([Y_1] - [Y_1]) + \frac{d[P_1]}{dt} \quad (3)$$

where  $D_1$  is the diffusion coefficient<sup>9</sup> for  $Y_1$ . Now if the association of  $Y_1$  and  $P_1$  to form the complex is rapid compared to either the diffusion

<sup>9</sup> The constant is perhaps more complicated since there is a set of membranes about the cyst so that more correctly, it should be multiplied by factors for membrane structure, partition coefficients etc.,—see Osterhout's papers (Osterhout 1932-33). For this analysis, however, this simple form of Fick's law suffices.

or the transformation of the complex, there would be an equilibrium at all times between  $Y_1$ ,  $P_1$ , and  $Y_1P_1$ , i.e.,

$$\frac{[Y_1][P_1]}{[Y_1P_1]} = K \quad (4)$$

The kinetic equation for the transformation would be

$$\frac{d^*[P_2]}{dt} = k_1[Y_1P_1] \quad (5)$$

in which  $^*[P_2]$  represents the concentration of  $P_2$  that would exist if  $P_2$  were not consumed by other reactions, the actual concentration of  $P_2$  can have no effect on the reaction since it is considered irreversible

Since for every molecule of  $P_1$  that enters into reaction, a molecule of  $Y_1P_1$  or  $P_2$  is formed, we may write the equation

$$[P_1] + [Y_1P_1] + ^*[P_2] = [P_1]_0 \quad (6)$$

where  $[P_1]_0$  is a constant assumed to be the concentration of  $P_1$  in the resting cyst

From equations 3, 4, and 6 it follows that

$$\frac{d^*[P_2]}{dt} = D_1(^*[Y_1] - ^*[Y_1]) \left( \frac{^*[Y_1] + K}{K} \right) - \left( \frac{^*[Y_1] + K}{K} + \frac{[P_1]_0 - ^*[P_2]}{^*[Y_1] + K} \right) \frac{d^*[Y_1]}{dt} \quad (7)$$

and from equations 4, 5, and 6 that

$$\frac{d^*[P_2]}{dt} = \frac{k_1^*[Y_1]([P_1]_0 - ^*[P_2])}{^*[Y_1] + K} \quad (8)$$

The solution of equations (7) and (8) is most easily made in limiting cases

*Case I*—Diffusion is limiting factor in rate of formation of  $P_2$ . In this case  $^*[Y_1] = 0$  and  $d^*[Y_1]/dt = 0$  for as soon as  $Y_1$  enters the site of reaction it is combined with  $P_1$  and goes to form  $P_2$ . Thus equation (7) reduces to  $d^*[P_2]/dt = D_1^*[Y_1]$  which may be immediately integrated to

$$^*[P_2] = D_1^*[Y_1]t = D_1\alpha_1[y\epsilon]t \quad (9)^{12}$$

<sup>12</sup> It is not so much the absolute value of  $^*[Y_1]$  that must remain small during the period in which diffusion is a controlling factor in order that  $d^*[P_2]/dt = D_1\alpha_1[y\epsilon]$  but that  $^*[Y_1]/^*[Y_1] < 1$ . Since the range of concentrations tested is very extensive, even though this ratio is not small for the lowest concentration, it is surely so for the majority of them

and the time  $t_2$  for production of the requisite amount of  $P_2$  will be inversely proportional to  $[y e]$

*Case II* —Reaction time is limiting factor in rate of formation of  $P_2$ . In this case, except for a negligible time close to  $t = 0$ ,  $[Y_1] = [Y_1] = \alpha [y e]$  and equation (8) becomes

$$\frac{d^*[P_2]}{dt} = \frac{k_1 \alpha_1 [y e]}{K_1 + \alpha_1 [y e]} ([P_1]_0 - [P_2]) \quad (10)$$

which integrates to

$$[P_2] = [P_1]_0 \left( 1 - e^{-\frac{k_1 \alpha_1 [y e]}{K_1 + [y e]} t} \right) \quad (11)$$

Now if  $\alpha_1 [y e] \ll K_1^{11}$  and the exponent as a whole is small, the expression reduces to

$$[P_2] = [P_1]_0 \frac{k_1 \alpha_1}{K_1} [y e] t \quad (12)$$

and again we have the time  $t_2$  inversely proportional to  $[y e]$

*Case III* —Diffusion time and reaction time of the same order. The analytical expressions for this case have not been determined directly but approximated by taking the time for  $[P_2]$  to reach  $[P_2]_2$  to be the sum of the time for diffusion and the time for reaction as found in cases I and II,  $t e$ ,

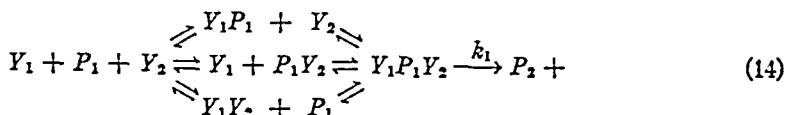
$$t_2 = \frac{[P_2]_2}{\alpha_1 [y e]} \left( \frac{1}{D_1} + \frac{K_1}{k_1 [P_1]_0} \right) \quad (13)$$

The range of temperatures over which both are of the same order happens to be small compared with the total temperature range so that we are not dependent on this case for a very wide temperature range

In this analysis we have started with a simple reaction, equation (2), and though it gives the proper relation between  $t_2$  and  $[y e]$ , equation (13), it is too simple, for it was shown by Thumann and Barker (1934) that an excystment medium (hay extract) could be separated into two fractions each containing an essential excystment compound, one with a high distribution coefficient between water and ether and the

<sup>11</sup> Barker and Taylor (1933) estimate the excystment compound need only be present in concentrations of the order of one part in ten to a hundred million parts of solution in order to bring about complete excystment

other with a low coefficient. Thus the reactions may be of the general form



For this case the equilibrium equations are

$$\begin{aligned} \frac{[Y_1][P_1]}{[Y_1 P_1]} &= K_1 & \frac{[P_1][Y_2]}{[P_1 Y_2]} &= K'_1 & \frac{[Y_1][Y_2]}{[Y_1 Y_2]} &= K''_1 \\ \frac{[Y_1 P_1][Y_2]}{[Y_1 P_1 Y_2]} &= K_2 & \frac{[Y_1][P_1 Y_2]}{[Y_1 P_1 Y_2]} &= K'_2 & \frac{[Y_1 Y_2][P_1]}{[Y_1 P_1 Y_2]} &= K''_2 \end{aligned} \quad (15a-f)$$

which may be combined to give

$$\frac{[Y_1][P_1][Y_2]}{[Y_1 P_1 Y_2]} = K_1 K_2 = K'_1 K'_2 = K''_1 K''_2 \quad (15g)$$

The equation for the transformation then becomes

$$\frac{d^*[P_2]}{dt} = k_1 [Y_1 P_1 Y_2] \quad (16)$$

and, since  $P_1$  now is converted into  $Y_1 P_1$  and  $Y_2 P_1$ , equation (6) must be modified to

$$[P_1] + [Y_1 P_1] + [P_1 Y_2] + [Y_1 P_1 Y_2] + ^*[P_2] = [P_1]_0 \quad (17)$$

From these equations we obtain

$$\frac{d^*[P_2]}{dt} = \frac{k_1 [Y_1][Y_2]([P_1]_0 - ^*[P_2])}{K_1 K_2 + K_2 [Y_1] + K'_2 [Y_2] + [Y_1 Y_2]} \quad (18)$$

The complete diffusion equation has not been written, but in the case in which diffusion of  $Y_1$  is a limiting factor and  $D_1 \ll D_2$ , where  $D_2$  is the diffusion coefficient for  $Y_2$ , we get the same equation as before, i.e.,  $d^*[P_2]/dt = D_1 \nabla^2 [Y_1]$  or  $^*[P_2] = D_1 \alpha_1 [y e] t$

In the case of reaction rate a limiting factor, equation (18) may be solved to give

$$^*[P_2] = [P_1]_0 \left( 1 - e^{-\frac{k_1 \alpha_1 \alpha_2 [y e]^2 t}{K_1 K_2 + (K_2 \alpha_1 + K'_2 \alpha_2) [y e] + \alpha_1 \alpha_2 [y e]^2}} \right) \quad (19)$$

and for inverse relation between  $t_2$  and  $[y e]$  it is required that  $K_2 \gg K_1$  with  $K_1 \ll 1$ . Thus in place of equation 13 we have the equation

$$t_2 = \frac{^*[P_2]_2}{\alpha_1 [y e]} \left( \frac{1}{D_1} + \frac{(K_2 \alpha_1 + K'_2 \alpha_2)}{k_1 \alpha_2 [P_1]_0} \right) \quad (20)$$



The deviations toward too short an excystment time for low concentrations that may be noted in Figs 3 and 4 can now be explained by

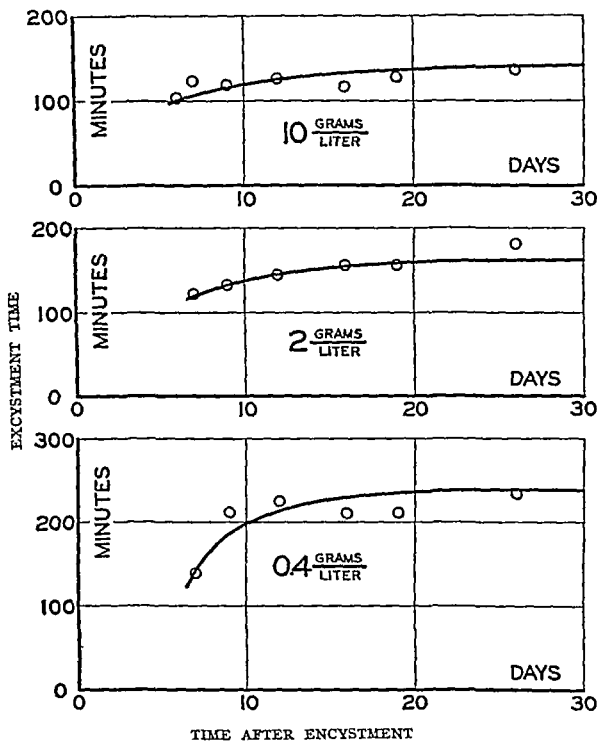


FIG 5 The change in excystment time with age of cyst Cellophane preparation during the 1st month all tests at 20 C.

$[Y_1]_0 \neq 0$  in the resting cyst This could not be true in the case of one active component in the extract since this component would react

to form  $P_2$ , but with two components required to form the complex, this condition could well exist. Further evidence that the concentration of the slowly diffusing substance in the resting cyst is not always negligible may be inferred from the increase in excystment time, especially with dilute extract, found during the first week or two after making the cyst-Cellophane preparation (Fig 5). This phenomenon finds a ready explanation if the slowly diffusing compound were gradually washed out during this period. Due to this increasing excystment time, tests made during the first 20 days, all at either 20°C or 17°C, have not entered into the averages of Table I. All observations made after 20 days and before 136 days, however, have been averaged, none omitted.

#### *Influence of Temperature on Excystment Processes*

In the preceding section it was shown that the period of excystment dependent on  $[ye]$  might be expected to follow the relation of equation (20) in which all symbols are constants except  $t_2$  and  $[ye]$  if the temperature is constant. Now if the temperature is a variable, the diffusion coefficient,  $D_1$ , would be expected to change in accordance with the Stokes-Einstein relation that  $D = RT/6\pi N\eta r^{1/2}$  where  $T$  is the absolute temperature,  $\eta$  the viscosity coefficient,  $R$  the gas constant,  $N$  Avogadro's number, and  $r$  the radius of the diffusing molecule. The velocity constant,  $k_1$ , would be expected to change in accordance with the Arrhenius equation,  $k = A \exp - \mu/RT$  and the equilibrium constants according to the van't Hoff reaction isochore equation that  $K = G \exp - \Delta H/RT$ , where  $\mu$  is a constant, the heat of activation,  $\Delta H$  the heat energy change per mole, and  $A$  and  $G$  constants.<sup>11</sup> Hence,

considering  $\Delta H$  negligible compared with heat of activation, equation (20) reduces to

$$t_2 = \frac{1}{[y\epsilon]} \left( \frac{\beta\eta}{T} + \gamma e^{\frac{\mu_1}{RT}} \right) \quad (21)$$

where  $\beta$  and  $\gamma$  are constants, each containing several unknown factors

Direct measurements of the viscosity coefficient,  $\eta$ , have not been made on *Colpoda*, but very fortunately the variations of  $\eta$  with temperature in another small protozoan, *Amoeba dubia*, have been made by Heilbrunn (1929a, 1929b) by the method of centrifuging naturally occurring granules. The constant  $\beta$  has been determined by calculating it for exact fit to Heilbrunn's curve at 17°C and again at 20°C (the reaction time is negligible at these temperatures) and then taking the mean of these two determinations. The values of the constants  $\ln \gamma$  and  $\mu$  have been determined from the values of  $B$  at 8°, 9°, 10°, and 11° by the method of least squares. Introducing these constants in the equation gives

$$t_2[y\epsilon] = B = \frac{4.16 \times 10^5 \eta}{T} + e^{\frac{221,644}{RT} - 333,922} \quad (22)$$

and it is this curve that is drawn in Fig. 6. The points are the average of  $B_a$  and  $B_b$  of Table II. The scales of  $\log t_2$  as ordinate and  $1/T$  as abscissa have been used since with these scales the data plot on straight lines if a chemical reaction is the limiting factor. The time  $t_2$  is in minutes if  $[y\epsilon]$  is measured in gm/liter and  $\eta$  is measured in poises.

The extraordinarily large value of 220,000 calories/mole for the energy of activation (equivalent to about 10 electron volts per molecule) for this reaction between the protoplasm and the excystment medium indicates that the activation probably involves several bonds. This is perhaps to be expected in biological systems where reactions may involve the very complex protein molecules and in this case may account for the stability of the encysted *Colpoda*.

The Arrhenius plot of the time for the excystment period independent of the concentration of the excystment extract is plotted in Fig. 7. It appears from inspection of this plot that there are at least two different reactions involved in this period and straight lines have been fitted by the method of least squares to the 8°, 9°, 10°, 11°, 12°, 13½° and 15° points and to the 17°, 20°, and 23° points. Above



That these values of  $\mu$  may be rather far from the true activation energies has been shown in an analysis of the case for two consecutive monomolecular reactions by Burton (1936). According to his analysis, the ratio between the reaction rates would have to differ by a factor of 49 at two points under consideration for the apparent values of activation energy to be within 10 per cent of the true value. In this case the ratio of the times for the separate reactions, taken near the midpoints of the two straight lines is only about 5 and according

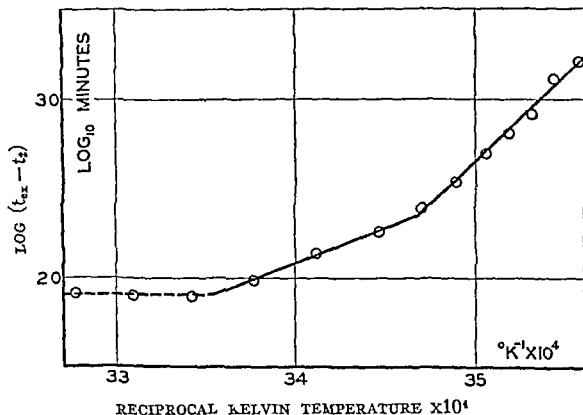


FIG. 7 Arrhenius plot of time for excystment processes independent of the concentration of the excystment medium

to the analysis for consecutive reactions, the true values of  $\mu$  could be of the order of 30 per cent from the observed values. However, if the reactions are limited by catalysts or are not consecutive but linked so that they run concurrently, the observed values of slope would give the correct activation energies.

It is thought by Hoagland (1937) from general considerations that there must exist controlling conditions so that the values of  $\mu$  truly characterize the individual reactions. He gives no examples or

mechanisms of such systems and even goes so far as to write that "no *simple* mechanism will give *sharp* breaks "

The value of 18,000 calories/mole is in the range found for many biological oxidations (Crozier, 1924-25, Lineweaver, Burk, and Horner, 1931-32, Stier, 1932-33, Crozier, Tang, and French, 1934-35, and Hoagland, 1935) and since it is known that oxygen is required for the excystment process (unpublished data), it is tentatively assumed that this reaction is an oxidation. If this is true, then the leveling of the curve at 25° may be due to the rate of transmission of oxygen becoming a limiting factor above this temperature

### *The Distribution of Excystment Times of Individual Organisms*

As noted in an earlier paragraph, the distribution of the log excystment times about the mean log excystment time is according to the normal distribution in all cases. The mean value of the standard deviation,  $\sigma$ , for each temperature and concentration is recorded in Table I and plotted in the graphs of Fig. 8. It is seen that  $\sigma$  shows no consistent change with temperature except for temperatures below 12°C though diffusion time and reaction times both change throughout the entire temperature range. The straight lines are the weighted averages for all temperatures above 12°C considering each test of equal significance. Each of the points which is shown on the graph may thus have a different weight.

The type of distribution<sup>14</sup> itself indicates that the principal factors causing deviation from the mean act throughout the excystment period, not during a part of it only. Further evidence that the dispersion is one of reaction rates rather than starting times is seen in that the diffusion time variations between 12° and 32° do not noticeably appear in the curves for  $\sigma$ . The break in the curves for  $\sigma$  at 12°C corresponds nicely to the point at which with lowering temperature the reaction with an ingredient of the yeast extract ceases to take a negligible length of time.

The concept which seems most consistent with these data is that the dispersion is principally dependent on the extent of the reaction between the excystment compound of the yeast extract and the proto-

<sup>14</sup> See footnote 5

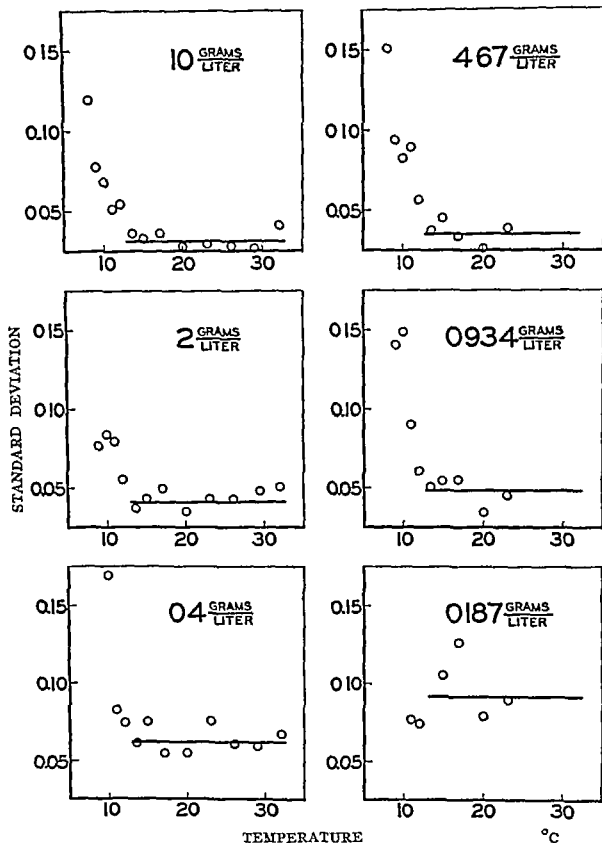


FIG 8 Influence of temperature on deviations of excystment times of individual organisms from the mean log excystment time.

plasm This reaction, being one which depends on both the protoplasm and its environment, takes a significant length of time only at temperatures below  $12^{\circ}\text{C}$  and, being the first, could influence all excystment reactions

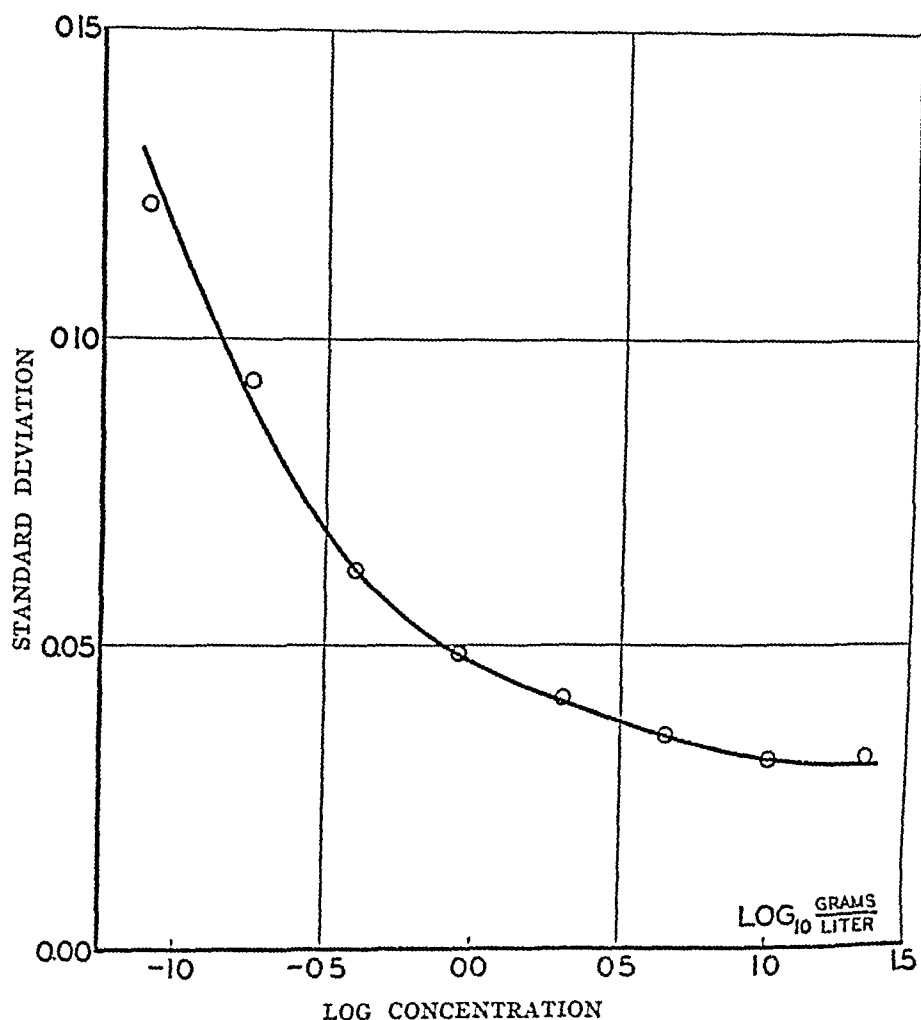


FIG 9 Influence of yeast extract concentration on deviations of excystment times of individual organisms from the mean log excystment time for the temperature range  $12^{\circ}\text{C} < \theta \leq 32^{\circ}\text{C}$

As the yeast extract concentration is increased, the deviation becomes asymptotic to a value of about 0.03 (Fig 9). It seems probable that this minimum deviation is characteristic of the particular pro-



paration used rather than of the excystment environment and could be lowered only by increased refinement of the encystment technique

#### DISCUSSION

In this paper the redifferentiation of *Colpoda duodenaria* from the resting cyst to the free swimming protozoan is shown to be a process which may be divided into periods, the first, a period dependent on diffusion through the protoplasm of some essential substance from the excystment medium followed by a chemical reaction with an activation energy of 220,000 calories/mole, and the second, a period controlled by two reactions, one with activation energy of 18,000 calories/mole dominant above 15°C and one with 44,000 calories/mole dominant below 15°C. At the present time the chemical compounds involved in this process are unknown, however, a generalized hypothetical system of chemical reactions, consistent in every detail with the chemophysical findings, is proposed. It is suggested that the first reaction with 220,000 calories/mole activation energy is the formation of an enzyme, that the reaction with 18,000 calories/mole activation energy is a catabolic oxidation, and that the reaction with 44,000 calories/mole activation energy is an anabolic reaction, linked to the catabolic reaction for its energy requirement or dependent on it for some compound. As long as this production of energy or some essential compound is adequate, the anabolic reaction is limited by its inherent reaction rate (a complex may be involved whose concentration is a constant, that of some catalyst, possibly the above enzyme). Above 15°C this condition is not fulfilled (the catalyst would be unsaturated and the concentration of the complex lower) so that the rate of anabolism depends on the rate of the catabolic oxidation.

The compound whose diffusion into the protoplasm is the first controlling factor in the excystment process is possibly the essential ether soluble substance partially separated from hay extract by Thumann and Barker (1934). They noted that the water residue after ether extraction caused excystment to take a long time compared with that taken when the ether fraction or the original medium was used. This water residue may be assumed to have only a small amount of the more ether soluble compound so that it corresponds to very dilute extract in diffusion time. The rapidly diffusing compo-

ment,  $Y_2$ , would then be the more water soluble constituent of Thimann and Barker's hay extract

The changes in diffusion time due to viscosity changes follow a rather peculiar curve and the excellent fit of this viscosity-temperature curve inferred from diffusion to the curve found by Heilbrunn (1929*a*) by direct measurement of the time for sedimentation of naturally occurring granules in centrifuged *Amoeba dubia* checks this relation in small protozoa from different sub-phyla, viz., Ciliata and Sarcodina. It may be noted that the fit would be still better if the curve were shifted by about  $1.5^\circ\text{C}$ . It was suggested by Heilbrunn (1929*a*) that the normal temperature environment may determine the position of the curve, his curve for *Amoeba dubia* being shifted several degrees (of the order of  $10^\circ\text{C}$ ) above that for *Cumingia* egg (Heilbrunn, 1924) which would be accustomed to the lower ocean temperatures. However, a  $-1.5^\circ$  shift from that of *Amoeba dubia* cultured at  $18^\circ\text{C}$  to that of *Colpoda duodenaria* cultured at  $20^\circ\text{C}$  is in the opposite direction.

The relation between the change in sensitivity to x-ray irradiation at a certain stage in the excystment process (Taylor, Brown, and Strickland, 1936) and the set of chemical reactions proposed from this study is not evident. In the x-ray experiments the conditions were such that the first period, diffusion and first chemical reaction, was complete in less than 10 minutes. The shift in effectiveness of x-rays in delaying excystment came at approximately 85 minutes after the start of excystment. Hence though this phenomenon cannot now be correlated with the reactions that control excystment rate, the reaction between the protoplasm and the yeast extract is evidently not the one blocked by the irradiation, the photosensitive condition persisting until relatively late in the excystment period independent of the concentration of the excystment medium.

#### SUMMARY

1 Extensive experimental data have been collected on the time required for the excystment process of the small ciliate *Colpoda duodenaria* throughout a range of temperatures of  $8^\circ$  through  $32^\circ\text{C}$  and a range of concentrations of yeast extract excystment media of 0.03 through  $22.4\text{ gm/liter}$ .

2 The excystment process has been separated into two periods, the first inversely proportional to the concentration of the yeast extract and the second independent of its concentration

3 The first excystment period has been found to depend on the time for diffusion through the protoplasm of a compound from the yeast extract and on the time for a chemical reaction with the extremely high energy of activation of 220,000 calories/mole

4 The changes in viscosity with temperature for this *Colpoda*, inferred from diffusion rate changes, have been found to be almost the same as those found by Heilbrunn for *Amoeba dubia* by the direct method of centrifuging granules

5 The second excystment period is shown to be controlled by reactions whose apparent activation energies are 44,000 calories/mole below 15°C and 18,000 calories/mole above 15°C, above 25°C this period is independent of temperature

6 The distribution of the log excystment times of individual organisms about the mean log excystment time is found to be independent of temperature except in the range where the reaction with highest activation energy takes a significant length of time, and to increase rapidly with decreasing temperatures in this range

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# ISOLATION, CRYSTALLIZATION, AND PROPERTIES OF SWINE PEPSINOGEN

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In 1882 Langley (1) noticed that a slightly alkaline extract of gastric mucosa contained a material which was not pepsin but which changed into pepsin upon acidification of the extract. This alkali stable material capable of being converted into pepsin is known as pepsinogen. Holter and Northrop (2) partially purified pepsinogen by fractional salt precipitation of an extract of swine stomach mucosae.

## *Method of Isolation*

The total pepsinogen in an average swine stomach mucosa is about a gram of which at least 90 per cent is located in that third of the mucosa known as the fundus. The minced fundus mucosae were extracted with a bicarbonate ammonium sulfate solution followed by precipitation of the soluble pepsinogen at a higher concentration of ammonium sulfate. By this one extraction and precipitation an 80 per cent yield of the total pepsinogen was obtained. The product was 50 times as pure as the starting material and half as pure as the final crystalline product, based on the protein nitrogen analyses. The material at this stage was nearly the same as the best preparations of Holter and Northrop (2). Further purification of pepsinogen by fractional salt precipitation was unsuccessful. The preparation still contained considerable quantities of carbohydrate as well as protein impurities which were removed by treatment with a copper hydroxide reagent.

When a washed copper hydroxide suspension at pH 6.0 was mixed with a solution of the pepsinogen described just above, practically all of the protein and carbohydrate materials were bound to the copper precipitate. Upon extracting this precipitate with pH 6.8

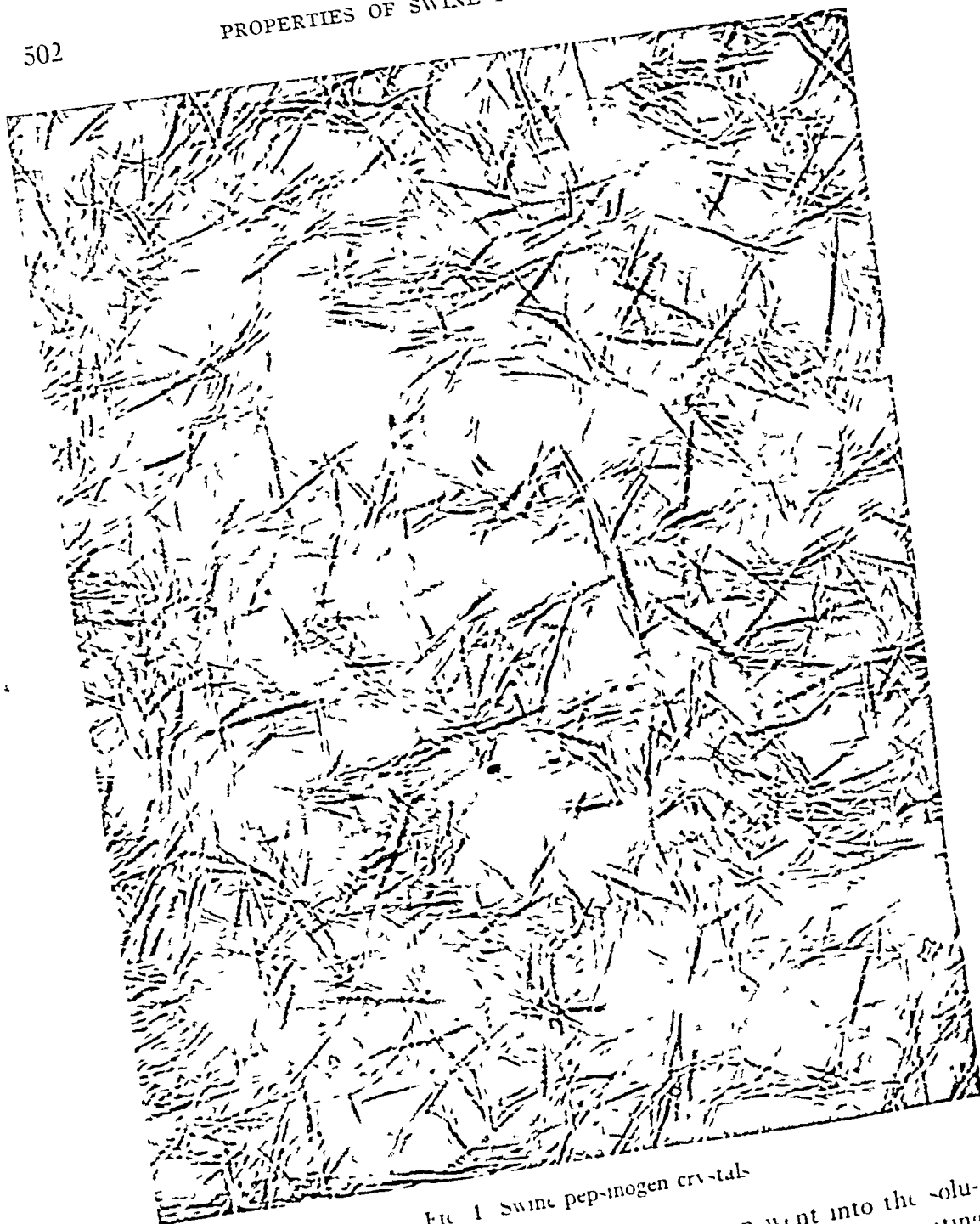


FIG. 1 Swine pep-inogen crystals

phosphate buffer 70 per cent of the pepsinogen went into the solution along with only a few per cent of the impurities. By repeating

this treatment with copper hydroxide a pepsinogen preparation was obtained which contained negligible quantities of carbohydrate and less than 5 per cent protein impurities. In fact no detectable improvement in the quality of the pepsinogen has been obtained with any subsequent treatment.

Crystallization of the pepsinogen after two treatments with the copper reagent was carried out by dissolving a 0.7 saturated ammonium sulfate filter cake in 9 volumes of 0.4 saturated ammonium sulfate M/10 pH 6.3 phosphate buffer and stirring the solution slowly at 10°C. In 6–24 hours the beaker was a swirling mass of very thin long needles of crystalline pepsinogen (Fig. 1).

### *Tests of Purity*

The first evidence of the purity of pepsinogen is a solubility experiment based on the phase rule which states that for a given solvent the solubility of a pure substance is independent of the quantity of solid phase, *i.e.*, the amount dissolved will not vary with the amount of solid. The solubility of pepsinogen in 0.55 saturated ammonium sulfate M/10 6.8 phosphate buffer was found to be constant over a 20 fold variation in the quantity of solid pepsinogen. The quantity of pepsinogen dissolved was determined in three different ways, namely, estimation of the dissolved protein nitrogen and the amount of pepsin produced upon acidification as estimated by the hemoglobin and rennet methods.

The remaining evidence concerning the purity of pepsinogen is the negative results of numerous experiments designed to separate the hypothetically pure pepsinogen from any impurity. Since the fundamental and characteristic property of pepsinogen is its quantitative conversion into pepsin and since the activity of pepsin can be determined with a precision of nearly 5 per cent, it was this property of pepsinogen (designated as "potential activity") that was examined during the following experiments. The fractionation experiments consist of changing either a small percentage or a large percentage of the pepsinogen by some procedure and then determining the potential activity per milligram of protein nitrogen in either the small percentage that was altered or in the small percentage left unchanged. If a protein impurity is present in the preparation before fractionation then it would be expected that some one of the fractionation

procedures would at least partially separate it from the pure pepsinogen and in doing so would raise the potential activity per milligram of protein nitrogen in the other fraction. The procedure used in the first experiment was fractional recrystallization in which both the crystals and the mother liquor were analyzed. It was to be expected that any impurity would tend to come out with the crystals or remain in solution in the mother liquor in some quantity other than just in proportion to the quantity of pepsinogen. Likewise, experiments were performed in which fractionation was carried out after partial heat and alkali denaturation and after partial reversal of completely heat and alkali denatured pepsinogen. In these experiments it was expected that an impurity would be separated into one fraction and would thus raise the potential activity per milligram of protein nitrogen of the other. However, in no case was there any consistent or definite evidence of a fraction having higher potential activity per milligram of protein nitrogen. It is concluded, therefore, that the pepsinogen as isolated, having withstood the available tests for protein purity, is a pure protein to the extent that the methods are precise, about 5 per cent.

### *Properties of Pepsinogen*

Table I contains a summary of the properties of pepsinogen and, for comparative purposes, the corresponding properties of pepsin have been included. In general it may be stated that those properties which depend for the most part on the whole molecule are much the same in the two proteins, whereas those properties which are more probably a function of particular groups or structural parts of the protein are in a number of instances different. Thus, the elementary analyses, molecular weights, absorption spectra, and optical rotation are qualitatively the same in pepsin and pepsinogen whereas the enzymatic activity, pH stability range, isoelectric point, amino nitrogen, titration curves, reversibility of heat and alkaline denaturation, and antigenicity (3) are quite different in the two proteins.

Denaturation, one of the most characteristic properties of proteins is so different in pepsin and pepsinogen as to be worthy of particular notice. Alkali denaturation of pepsin takes place at appreciable rates in solutions more alkaline than pH 6.0. This has recently been



TABLE I  
*Comparison of Properties of Some Pepsin and Pepsinogen*

Comparison of Properties of Swine Pepsin and Pepsinogen

Property	Crystalline form	Catalytic activity	Specific activity or specific potential activity [P U] <sub>Hb</sub> /mg P.N	23 C. [α] <sub>D</sub> pH 5.6 per gram of dry protein	I.E.P							
Pepsin	Hexagonal bipyramids	+	0.26	-72	pH 2.7							
Pepsinogen	Needles	-	0.22	-62	pH 3.8							
Property	Molecular weight (osmotic pressure)	NH <sub>2</sub> -N per cent of T.N	Reversibility of denaturation	Hydration or molecular shape								
				Hydrated or non spherical	Non hydrated and spherical							
Pepsin	38 000 ± 3 000	1.4	1.0 per cent									
Pepsinogen	42 000 ± 3 000	4	80-100 per cent									
Property	pH-stability	pK of alkaline inactivation at 25 C	Tyrosine-tryptophane content at pH 11	at pH 8.0	Absorption spectrum	Elementary analyses per cent of dry protein						
						C	H	N Dumas	N Kjeldahl	S	P	Cl
Pepsin	pH 2.0-6.0	7.0	9.3	5.5	Same	51.7	6.8	14.7	14.0	0.45	0.09	0.00
Pepsinogen	pH 6.0-9.0	9.4	8.6	3.2	Same	52.8	6.9	14.2	13.9	0.4	0.08	0.00

studied in considerable detail by Steinhardt (4) The reaction follows a simple unimolecular course with an increase in rate as the pH increases and in concentrated solutions the denaturation is not reversible Although a detailed study has not been made heat denaturation is not readily reversible and Northrop (5) has shown that it also followed a unimolecular course Pepsinogen, on the other hand, although denatured by heat or in solutions more alkaline than pH 8.5, is denatured in a completely reversible manner After complete denaturation, as demonstrated by its insolubility in salt solutions in which the native form is soluble and by the fact that the protein is not converted into pepsin when mixed with acid, the denaturation may be completely reversed and the pepsinogen shown to be native by the same criteria as were used to test for denaturation Denaturation of pepsinogen is similar to the denaturation of trypsin by heat (6) or of hemoglobin by salicylate (7) That the denaturation of pepsinogen is a true equilibrium reaction was established by the fact that at any temperature or pH in heat or alkali denaturation the quantity denatured is the same whether that temperature or pH be approached from the higher or lower value, *i.e.* the quantity of native or denatured protein is the same when the conditions are approached from the completely native or completely denatured side of the equilibrium Upon long exposure of pepsinogen to the denaturing agents the reversibly denatured material is slowly changed into an irreversible form The rate of this change is increased by an increase in temperature or pH and, in the case of the change caused by heat, the reaction follows the course of a unimolecular reaction

It may be seen from the titration curves of pepsin and pepsinogen that the total number of groups titrating between pH 6.0 and pH 12.0 is nearly the same in the two proteins but the pH at which the groups titrate is different, *i.e.*, the slope at any pH is not the same for the two proteins However, the pH at which the groups titrate coincides with the pH at which the corresponding protein is rapidly denatured

When solutions of pepsinogen are made more acid than pH 6.0 the protein is converted into pepsin This conversion of pepsinogen into pepsin has been examined from two angles, the kinetic and chemical In the kinetic study it was found that between pH 4.5-

5.0 the course of the conversion is accurately described by a simple autocatalytic equation. That the reaction is autocatalytic means that a product of the reaction, in this case pepsin, catalyzes the reaction and therefore produces more of itself. The reaction is at least partially autocatalytic at pH 0.0 as shown by an increase in the rate of conversion upon addition of pepsin. However, in solutions more acid than pH 4.0 the reaction appears to deviate from the simple autocatalytic. The rate of conversion is greatly increased as the pH decreases with a maximum near pH 1.0. The rate decreases beyond this maximum as the pH decreases and this decrease in activity does not appear to be an acid denaturing effect upon either pepsin or pepsinogen. The presence of salts in the medium has an accelerating effect upon the conversion. The concentration and charge or valency of the ions of the salt are variables which affect the rate. Kunitz and Northrop (8) have noticed a somewhat similar effect of salt on the autocatalytic conversion of trypsinogen into trypsin.

A chemical study of the conversion has shown that as pepsin is formed from pepsinogen there is a concomitant cleavage of the pepsinogen molecule. 15–20 per cent of the pepsinogen nitrogen appears in the solution as non protein nitrogen, *i.e.*, nitrogen not precipitated by hot 2.5 per cent trichloroacetic acid. It was demonstrated that this appearance of non protein nitrogen in the solution was not the complete digestion of part of the pepsinogen by pepsin by the fact that addition of three times as much pepsin as pepsinogen resulted in the production of the same quantity of non protein nitrogen upon activation as did the control where activation was spontaneous. It could be supposed that in spite of the tests for purity which showed the pepsinogen preparations to contain little or no protein impurity that there was 15 per cent of a foreign protein which consistently resisted fractionation. This was made less likely by some experiments which showed that under widely different conditions of activation the change in non protein nitrogen varies linearly with the increase in activity and that under these same varied conditions the increase in non protein nitrogen and activity stop at the same time.

Part, if not all, of this 15–20 per cent non protein nitrogen is capable of acting as a dissociable inhibitor with pepsin. Its effect is most pronounced at pH 5.8 where the rennet activity of pepsin is

measured. Dissociation of the inhibitor pepsin complex takes place in acid so that there is no complicating effect on the hemoglobin activity measurement of activation mixtures. The amino nitrogen of an activation mixture is larger than the original pepsinogen by about nine amino groups per molecule of pepsinogen. It is to be expected that the conversion of pepsinogen into pepsin would result in an increase in amino nitrogen since pepsin is catalyzing the reaction and no linkage other than the peptide linkage is known to be split by pepsin.

It appears then, that upon acidification of a solution of pepsinogen an enzymatically inactive, reversibly denaturable protein with an isoelectric point of pH 3.8 is split at probably not more than nine peptide linkages to produce pepsin, an active, irreversibly denaturable protein isoelectric at pH 2.7, and a non-protein nitrogenous fragment which contains 15–20 per cent of the original pepsinogen nitrogen.

#### EXPERIMENTAL RESULTS

##### *Preparation of Crystalline Pepsinogen*

A detailed account of a typical preparation is presented in Table II. The text of this section includes a description of the first step in the preparation and a discussion of some of the procedures and materials used in the remainder of the preparation exemplified in Table II.

The mucosae used throughout this work have been prepared from stomachs of freshly killed swine. The stomach is removed immediately after death, turned inside out, and the contents washed away with cold water. No ill effects will be noticed if the stomachs remain after washing for as much as 5 hours at room temperature but a longer time is to be avoided.

The fundus part is the only desirable portion of the stomach and is accordingly cut out from the remainder with scissors. The fundus is pink or darker in color than the surrounding tissue and may easily be recognized when the stomach is inside out.

The layer of muscle tissue must be separated from the mucosa of the fundus. This is most expediently carried out by first carefully cutting the connecting tissue to allow one to grip the layers separately and then pulling in opposite directions. The fundus mucosa is then placed upon a flat surface and the clinging slime or mucous scraped off with the aid of a microscope slide or any piece of glass having a straight edge. This yellow mucous, if not removed, greatly retards the subsequent purification procedures. Analyses of swine gastric mucous have recently been made (9). Removal of the slime is followed by a rinse in cold water. The

mucosae are now ready for use and may be either minced and extracted immediately or stored in a freezing ice box. Fundus mucosae which had been stored at  $-13^{\circ}\text{C}$  for as long as 6 months exhibited no detectable difference from the freshly prepared ones with regard to either the handling or to the pepsinogen content.

A description of the preparation of the copper hydroxide suspension is to be found in the section of this paper devoted to Experimental methods. The pH of the copper hydroxide suspension is a factor of considerable importance. It must be  $\text{pH } 6.0 \pm 0.2$  as indicated by brom thymol blue and methyl red. Sulfuric acid and sodium hydroxide may be used to adjust the pH of the suspension to pH 6.0. If the pH is too acid (pH 5.5 or less) activation of pepsinogen takes place and if too alkaline (over pH 6.3) the reaction between the copper suspension and the pepsinogen solution does not give the desired results. Titration of the pepsinogen solution to  $\text{pH } 6.0 \pm 0.2$  is also necessary for the same reasons. This titration is best carried out with 4 M pH 5.0 acetate buffer so that at no time is any portion of the solution below pH 5.0.

Pepsinogen of 1.0–1.5 mg protein nitrogen per ml. concentration is completely removed from solution at pH 6.0 when mixed with an equal volume of washed copper hydroxide suspension of about molar concentration. Most of the soluble carbohydrate goes with the pepsinogen and the copper hydroxide.

The phosphate-copper pepsinogen suspension usually has an acidity of pH 6.5 to brom thymol blue. After filtration of this suspension and washing the residue as directed, most of the pepsinogen is found in the filtrate along with only small amounts of impurities.

### *Crystallization*

After the second or last copper treatment followed by filtration with 5 per cent Filter Cel, the pepsinogen is precipitated in the following manner: the solution is titrated to pH 6.0 with 4 M pH 5.0 acetate and brought to about 0.4 saturation by the addition of 242 gm. solid ammonium sulfate per liter of solution. Now with slow but constant stirring a volume of saturated ammonium sulfate equal to the volume of the 0.4 saturated ammonium sulfate protein solution is added through a capillary tube dipping below the surface of the liquid. Instead of filtering, the suspension is now stored for 1 or 2 days at  $5-10^{\circ}\text{C}$ . After this time the suspension is filtered with suction on a C S and S No. 575 hardened filter paper without the aid of any Celite and the residue is then stirred in 9 volumes of 0.40–0.42 saturated ammonium sulfate in  $\text{M}/10$  pH 6.25 phosphate buffer at  $10^{\circ}\text{C}$ . The precipitate usually dissolves but it is very sensitive to the ammonium sulfate concentration. In the event it does not dissolve  $\text{M}/10$  pH 6.25 phosphate should be added dropwise until the precipitate goes into solution. Although pH 6.25 phosphate is used the pH of the crystallizing mixture is about pH 5.0 as determined by both the quinhydrone and glass electrodes. This change is due to the effect of the high concentration of salt. If this solution is now stirred slowly but constantly the pepsinogen

TABLE 11

## Preparation of Crystalline Pepsinogen from Swine Gastric Mucosa

## PROPERTIES OF SWINE PEPSINOGEN

TABLE 2

PROPERTIES OF SWINE

Preparation of Crystalline Pepsinogen from Swine Gastric Mucosa

No	Procedure, details of preparation, etc	Time of handling hrs	Vol ml	Protein nitro- gen mg/ml	Potential p U/gm*			Carbohydrate (as glucose)		
					1/ml	1/mg P <sub>N</sub>	total of acid nit	mg/ml	1/ p U/gm	
1	3100 gm of twice minced, prepared, frozen swine gastric fundus mucosa mixed with 1 time its weight of 0.45 sat ammonium sulfate in M/10 sodium bicarbonate, reduced 1 hr	1 5	16000	0.67	0.012	0.07	670	0.65	11.0	
2	To No 1 was added 100 gm fine "filter Cel" and 50 gm coarse (115 flow) Cel per liter of 0.15 sat ammonium sulfate solution used. Stirred 15 min and filtered on 30 cm Buchner funnel with Whatman No 3 paper covered with a thin cake of coarse Cel. Filtrate clear	2 0	13000	0.37	0.018	0.13	630	0.21	5.0	
3	cake was neatly dry it was washed on funnel twice with 400-500 ml of 0.15 sat ammonium sulfate in M/10 sodium bicarbonate, residue discarded, filtrate and washings No 2 was brought to 0.68 sat by addition of 180 gm solid ammonium sulfate, per liter. Solid ammonium sulfate allowed to stand 10 min in solution before stirring and then stirred slowly. The suspension allowed to settle overnight or filtered immediately after addition of 50-100 gm coarse Cel. Discard filtrate	3 0 5	14000		0.0022		30	5	0.16	73.0
4	Above residue dissolved in water and Cel filtered off. Above residue on the funnel until a sample of the washings showed no protein present. Filtrate and washings were combined	4 0 3-0 5	1500	1.3	0.15	0.12	530	79	0.3	2.0

No 4 was titrated to pH 6.0 $\pm$ 0.2 (yellow to methyl red and to brom thymol blue) with 4 M pH 4.65 acetate buffer and mixed with equal vol M/1 copper hydroxide suspension previously titrated to pH 6.0 Mixture stirred a few minutes and filtered on large Buchner funnels Filtrate contained no protein as shown by heating 3 ml of filtrate with 3 ml of 10 per cent trichloroacetic acid If protein is found more copper suspension must be added and refiltered Final filtrates were discarded Filtrate Copper residue from No 5 was stirred to a smooth paste with a vol of M/10 pH 6.8 phosphate equal to that vol occupied by the protein solution just before mixing with the copper suspension This is also the vol of copper suspension used In this experiment it was 3500 ml The residue was filtered and washed twice with 250 ml M/10 pH 6.8 phosphate Residue discarded and the filtrates and washings combined Filtrates To No 6 was added 5 per cent fine (Filter Cel) Cel stirred and filtered residue washed twice with 1 vol M/10 pH 6.8 phosphate equal in ml to the weight in gm of fine Cel used. Residue discarded Filtrate	5	2-3	6500	0.003	20	3	0.053	17	0	
	6	1.5	5000	0.33	0.075	0.23	375	55	0.03	0.4
	7	0.25	5100	0.33	0.071	0.20	360	54	0.017	0.24

\* These values represent the pepsin activity after complete activation of aliquots of pepsinogen. For details of measurements see the section on Experimental methods.

TABLE II—*Concluded*

Procedure, details of preparation, etc	No	Time of handling hrs	Vol ml	Protein nitro- gen mg/ml	Potential [P U] Hb*			Carbohydrate (as Glucose)	
					1/mg P N	total	per cent of origi- nal	mg/ml	1/[P U] Hb
No 7 brought to 0.7 sat by addition of 47.4 gm of solid ammonium sulfate per liter. 50-100 gm coarse Cel added and suspension filtered. Filtrate discarded and residue stirred with water and separated from the Celite as described in No 4. Solution should be diluted with water until the concentration of P N/ml is about 1 mg. Solution titrated to pH 6.0 as before and copper hydroxide treatment repeated as well as the treatment with 5 per cent fine Cel as described in Nos 5, 6, and 7. Solution brought to 0.4 sat by adding 242 gm solid ammonium sulfate per liter. An equal vol of sat ammonium sulfate solution was added through a capillary tubing. Suspension should be stirred slowly but constantly. The protein was allowed to stand 2 days at 10°C and the protein was analyzed <i>without Celite filter aids</i> on a Buchner with hardened filter paper. (A sample of residue was analyzed and figures were calculated for the entire quantity)		8	6-8 and 8 stand- ing			0.25	320	48	0.03
No 8 as 1.07 sat ammonium sulfate filter cake was stirred with 9 vol 0.40 sat ammonium sulfate solution in M/10 pH 6.25 phosphate at 10°C. The solution was almost clear, containing dirt and shreds of filter paper and was therefore filtered. Solution on stirring slowly at 10°C for a few hours became opalescent and a silliness appeared which grew heavier. After stirring 2 days suspension filtered. Filtrate Crystal cake dissolved		9		460	2.1	0.43	0.21	200	30
		10		126	3.5	0.74	0.21	94	14



will crystallize out slowly in long, very thin, fiber like needles. That crystallization has taken place is first apparent from the silkiness or swirl of the precipitate in the pepsinogen solution. If there is a precipitate but no swirling when stirred, microscopic examination will probably show either typical amorphous material or large spheroids. This condition is brought about by either too high a concentration of ammonium sulfate or incorrect hydrogen ion concentration. It indicates that the pepsinogen has come out too fast. If no precipitate appears in 12-24 hours either or both reasons may be responsible. The ammonium sulfate concentration may be too low or activation of some pepsinogen may have taken place resulting in a mixture of pepsin and pepsinogen from which pepsinogen would not crystallize.

Once crystallization takes place the system is allowed to remain unchanged for 24-36 hours. The crystals may then be filtered off or, if a larger yield is desired, the suspension is poured into an evaporating dish, stirred gently, and a stream of air passed over the surface. Other methods of forcing crystallization have not been successful.

Fig. 1 is a photograph of pepsinogen crystals. The crystals as they first come out are very thin or narrow and may be missed even by microscopic examination. The best identification of their being crystals is the swirl of a stirred suspension. At times the crystals are so small in diameter that they pass through the filter papers at first and then plug the paper so that filtration is very slow. Although Celite products help somewhat it still required 6-24 hours to filter a few hundred milliliters of suspension. Centrifugation has not been more successful.

### *Recrystallization*

Filter cakes of crystalline pepsinogen differ so radically in their protein content, due to difficulties in filtration, that it is impracticable to outline a definite scheme for recrystallization. In general the procedure is that used in the original crystallization except that precautions are taken to inactivate any pepsin which may have formed. Since activation is autocatalytic the amount of pepsin present must be minimal if crystallization of pepsinogen is to take place. This is most easily accomplished by making the pepsinogen alkaline just before commencing recrystallization. The simplest scheme is to dissolve the crystalline filter cake in the minimal amount of cold water, titrate it to pH 8.3 (just pink to 0.5 per cent phenolphthalein), warm to 40°C for 5-10 minutes, cool to 10°C and titrate to pH 6.2 (greenish yellow to brom thymol blue) with 4 M pH 4.65 acetate, and then add amounts of saturated ammonium sulfate and M/5 pH 6.25 phosphate to bring the final concentration of protein to 4-5 mg per ml and the ammonium sulfate concentration to 0.40-0.43 saturated. If the solution is not clear it should be filtered. Crystallization should proceed as in the original crystallization procedure. If it does not crystallize, precipitate the protein by slowly adding an equal volume of saturated ammonium sulfate and treat as in the scheme for the original crystallization.

### Storage

Pepsinogen may be kept as a 0.7 saturated ammonium sulfate filter cake in a closed container at 5–10°C. The writer has also found that no loss of activity occurs if a dialyzed solution of pepsinogen is frozen in carbon dioxide snow ("Dry Ice") and then dried *in vacuo* below 0°C. Although it is too soon to speak from experience with this protein it is to be expected that as a dry powder it will keep better than as a filter cake.

### Tests of Purity

*Solubility Experiment*—The solubility is represented graphically in Fig. 2.

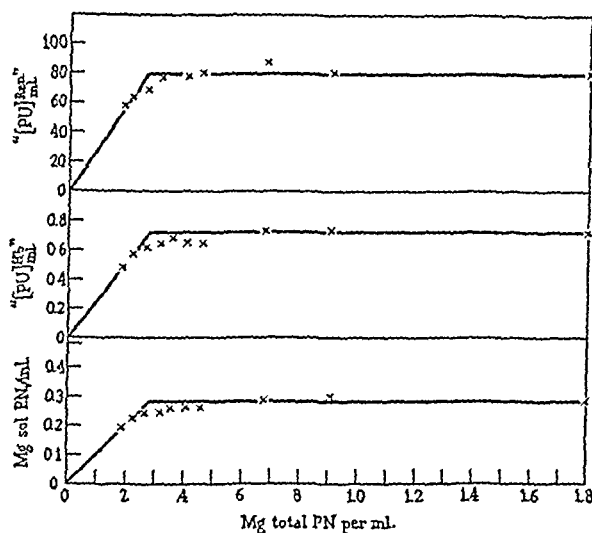


FIG. 2 Solubility of pepsinogen in 0.55 saturated ammonium sulfate— $M/10$  pH 6.8 phosphate at 21°C.

### Experimental Procedure

The solubility experiments were carried out at 21°C after first bringing to equilibrium the filter cake of twice crystallized pepsinogen and the solvent ( $M/50$  pH 6.8 phosphate — 0.55 saturated ammonium sulfate). This was accomplished by stirring the cake with a succession of 100 ml aliquots of the solvent until three consecutive filtrates were the same with respect to activity and protein nitrogen. Different amounts of the suspension were then added to a series of tubes and supplementary amounts of solvent were added to each tube to bring them to the same volume. 2.25 ml of 0.0444  $M$  phosphate buffer was then added to each tube which brought about solution of the precipitate. With rapid stirring 2.75 ml of saturated ammonium sulfate was added. The suspensions were filtered and

aliquots of the filtrates activated and analyzed for activity by the hemoglobin and rennet methods. Other aliquots of the filtrates were analyzed for protein nitrogen after diluting to contain approximately 0.05 mg protein nitrogen per ml. The analysis was carried out by adding 1.0 ml of the diluted solution to 10.0 ml of boiling 2.5 per cent trichloroacetic acid and measuring the quantity of precipitate formed in a Duboscq type photoelectric colorimeter. A solution of copper sulfate was used as the standard in the colorimeter and a reference curve was obtained using known quantities of pepsinogen similarly treated.

TABLE III  
*Fractional Crystallization of Pepsinogen*

Material analyzed	Protein calculated from P-N analyses	[P U] <sup>Hb</sup> / mg P-N	$[\alpha]_{D25}^C$ , pH 6.3 per gm. protein	Optical rotation / [P U] <sup>Hb</sup> / ml.
	gms			
Mother liquor of 1st crystallization	4	0.22	-67	2.1
Filter cake " "	13	0.21	-58	2.0
Mother liquor of 2nd crystallization	1	0.21		
Filter cake " "	12	0.23	-66	2.0
Mother liquor of 3rd crystallization†	4	0.25	-71	2.0
Filter cake	5	0.23	-67	2.0

\* This figure is the ratio of the optical rotation of a solution (not specific optical rotation  $[\alpha]$ ) to the hemoglobin activity per milliliter of the same solution. This ratio does not depend upon the nitrogen measurement as do the other two values.

† Analyses of this mother liquor revealed that activation had taken place to a high degree. This is indicated also in the high  $[\alpha]_D$  and [P U]<sup>Hb</sup> / mg P-N.

*Fractionation Experiments*—Fractionation of pepsinogen was carried out by a number of procedures which are summarized as follows.

A—Three fractional recrystallizations in which all fractions were analyzed for protein nitrogen, hemoglobin activity, and optical rotation. These results are to be found in Table III.

B—Salt fractionation after partial heat denaturation at pH 7.0 and 55°C, 60°C, and 65°C.

C—Salt fractionation after partial reversal at pH 7.0 and 55°C, 60°C, and 65°C of completely (70°C) denatured pepsinogen.

D—Salt fractionation after partial denaturation at 25°C and pH 9.1, 9.4, and 9.8.

*E*—Salt fractionation after partial reversal at 25°C and pH 9.5, 8.5, and 8.3 of completely (pH 10.5) denatured pepsinogen

Fractions in B, C, D, and E, were analyzed for protein nitrogen and hemoglobin activity and the results are given in Table IV. These experiments are a part of those described in the sections on *Reversible heat denaturation* and *Reversible alkali denaturation*.

TABLE IV  
*Fractionation of Pepsinogen*

Conditions or procedures in fractionation		Original	Potential P U <sub>1</sub> mg P N
		<i>per cent</i>	
B			
	Heat denaturation at 55°C	87.0	0.22
	" " " 60°C	46.0	0.20
	" " " 65°C	8.0	0.21
C	Reversal of heat (70°C) denaturation at 65°C	4.5	0.19
	" " " " " 60°C	40.0	0.20
	" " " " " 55°C	74.0	0.21
	" " " " " 50°C	93.0	0.22
D			
	Alkali denaturation at pH 9.1	78.0	0.22
	" " " pH 9.4	52.0	0.20
	" " " pH 9.8	13.0	0.22
E	Reversal of pH 10.5 denaturation at pH 9.5	44.0	0.22
	" " " " " pH 8.5	78.0	0.22
	" " " " " for 15 seconds	24.0	0.23
	at pH 8.3		

*Properties of Pepsinogen*

*Elementary Analyses*

The analyses are given in Table V. The high ash content in all preparations was mostly sodium and magnesium which remained combined with the protein on the alkaline side of the isoelectric point, even on long dialysis.

The percentage sulfur is less than that reported by Northrop (5) but it is nearly the same for pepsin and pepsinogen.

*Pepsin from Pepsinogen*

It is of primary importance that the material isolated and called pepsinogen yield pepsin upon activation. Consequently crystalline pepsinogen was activated and the pepsin thus formed twice crystallized. This material was analyzed along with a sample of crystalline pepsin prepared in the manner described by Northrop (5) from commercial pepsin.

TABLE V  
Elementary Analyses\*  
Dialyzed and Dried in vacuo at 80°C

Materials	C	H	N Dumas	N† Kjeldahl	S	P	Cl	Ash
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
2 × crystallized Cudahy pepsin	51.74	6.54	14.76	13.8	0.49	0.15	0.00	2.17
2 × crystallized Parke Davis pepsin	51.70	6.58	14.83	14.1	0.51	0.11	0.00	1.70
						0.09†		
2 × crystallized pepsin from pepsinogen	51.61	6.86	14.63	13.9	0.42	0.09	0.00	1.78
						0.09†		
Uncrystallized pepsinogen	52.80	6.88	15.92	15.1	0.37	0.09	0.00	1.72
						0.084†		
Uncrystallized pepsinogen			14.40	13.8		0.084†		
3 × crystallized pepsinogen			14.45	14.3				
3 × crystallized (Na) pepsinogen			14.00	14.00				
3 × crystallized No. 9 pepsinogen			13.59	13.4				

\* Unless otherwise noted the analyses were micro analyses and were made by Dr. A. Elek of Dr. P. A. Levene's laboratories.

† The Kjeldahl nitrogen estimations were carried out as previously described (11).

‡ Carried out by the writer using the method of Sørensen (10).

Most of the analyses and comparisons are to be found in the tables to follow under the specific properties. The crystalline form, optical rotation, and specific activities of the pepsin prepared from crystalline pepsinogen were similar to those properties of crystalline pepsins from commercial preparations. This is, of course, to be expected since the commercial pepsin is swine pepsin and came originally from swine pepsinogen.

*Proteolytic Activity of Pepsinogen*

It was of interest to see if pepsinogen had any proteolytic activity. Most pepsin activity measurements are carried out under conditions which would normally activate pepsinogen rapidly. However, two methods of estimation, the rennet and gelatin viscosity methods are carried out at pH 5.8 and 4.7 respectively. At these acidities the extent of activation of pepsinogen is not appreciable in the time required for measurement.

TABLE VI  
*Specific Activities*

Materials	Methods*			
	Hemoglobin Hb P U /mg P N	Rennet Ren P U /mg P N	Gel viscosity Gel V- P U /mg P N	Edestin N P N Ed sol P U /mg P N
5 × crystallized Parke Davis pepsin	0.21	475	13	0.50
3 × crystallized Parke Davis pepsin 9-19-33	0.22	410	18	0.44
2 × crystallized Cudahy pepsin 12-7-33	0.30	435	33	0.57
3 × crystallized Cudahy pepsin 43-12	0.26	470	33	0.55
Crystalline pepsin from crystalline pepsinogen	0.26	360	12	0.48
3 × crystallized pepsinogen		<1	<1	
Specific potential activities† of pepsinogen preparations				
3 × crystallized pepsinogen	0.20	275	17	0.42
Average of 10 preparations of pepsinogen	0.22	250-300		
3 copper treatment but uncrystallized pepsinogen	0.21	270	17	0.46

\* With the exception of the rennet method the methods were carried out as described by Northrop (12).

† Described in detail in the section devoted to Experimental methods.

‡ Explained in the text of this paper.

As seen from Table VI, pepsinogen is practically inactive proteolytically, as measured by its milk clotting activity and its action on gelatin.

*Reversible Heat Denaturation*

In the absence of salt, pepsinogen solutions may be heated to 100°C without the formation of a visible precipitate. Upon cooling the

solution and analyzing for pepsinogen it is found that a large proportion of the pepsinogen is indistinguishable from the pepsinogen before heating. That heating to 70–100°C actually denatures pepsinogen is indicated by the fact that a salt solution (M/1 magnesium sulfate) just sufficient to precipitate the pepsinogen above 70°C has no precipitating effect on the pepsinogen at 50°C, nor upon the “reversed” pepsinogen. Also the soluble denatured protein is not converted into pepsin upon acidification. Two independent methods of estimating the extent of denaturation were based on these facts and were used in the experiments shown in Fig. 3.

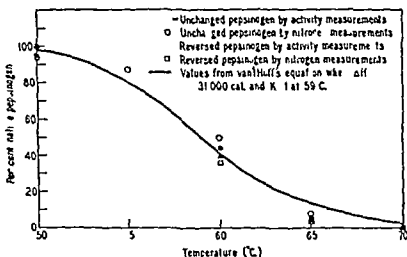


FIG. 3 Effect of temperature on the equilibrium between native and denatured pepsinogen at pH 7.0

### Experimental Procedure

**Pepsinogen Solution**—1 mg protein nitrogen per ml of a dialyzed pepsinogen preparation at pH 7.0

**Procedure I**—Approaching from the native or “activatable” side of the equilibrium to each of two 30 ml test tubes was added 5 ml. of the pepsinogen solution. These tubes were kept in a water bath at the desired temperature (50°C, 55°C, 60°C, 65°C or 70°C) for 10 minutes after which the solution in one tube was mixed with 5 ml. of 2 M magnesium sulfate at the same temperature, while the solution in the other tube was mixed with 5 ml. of N/20 hydrochloric acid also at the same temperature. The solution containing the magnesium sulfate was filtered and an analysis for nitrogen by the Kjeldahl method was performed on the clear filtrate. The solution to which had been added the N/20 hydrochloric acid was allowed to stand at 35°C for 5 minutes after which it was diluted and activity measurements performed by the hemoglobin method.

**II**—In approaching from the denatured or “unactivatable” side of the equilibrium two 5 ml portions of the pepsinogen solution were heated to 70°C for 5 minutes followed by cooling to the desired (50°C, 55°C, 60°C, 65°C) temperature.

for 5 minutes after which 1 aliquot was mixed with 5 ml of 2 M magnesium sulfate while the other was mixed with N/20 hydrochloric acid. Both of these reagents were kept at the temperature to which the protein solution had been cooled. The solutions were then analyzed as described in I for the solutions which were heated to the temperature for 10 minutes.

At about 59°C pH 7.0 and with 1.0 mg protein nitrogen per ml the denaturation equilibrium of pepsinogen is near the mid-point, *i.e.*, half the protein is denatured and half is native. The value of  $\Delta H$ , the energy of the reaction, taken from van't Hoff's equation

$$\ln K = -\frac{\Delta H}{RT} + C$$

is 31,000 calories in the equilibrium shown in Fig. 3

#### *Secondary Heat Change*

Heating to 100°C gradually converts reversibly denatured pepsinogen into an irreversible form. This may be seen graphically in Fig. 4

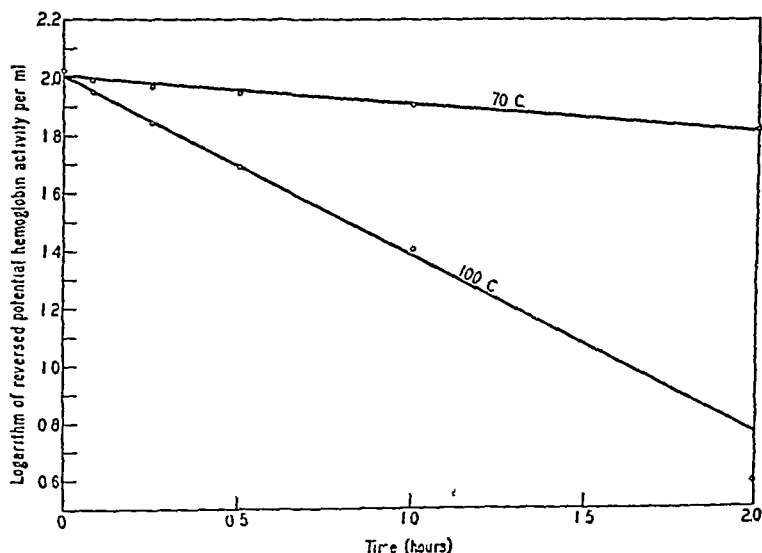


FIG. 4 Rate of change of reversibly denatured pepsinogen into an irreversible form at 70°C and 100°C

where the logarithm of the reversible potential hemoglobin activity is plotted against the time. It is apparent that whatever the nature of the change it follows a first order reaction.



### Experimental Procedure

Two 15 ml aliquots of a dialyzed pepsinogen solution at pH 7.0 containing 1.0 mg protein nitrogen per ml were heated at 70°C and 100°C. At intervals of time 2 ml samples were removed, cooled to 35°C for 30 minutes, followed by addition of 2 ml of N/20 hydrochloric acid. After 10 minutes allowed for activation the samples were diluted and the activity determined by the hemoglobin method.

### Reversible Alkali Denaturation

Alkali denaturation of pepsinogen is a true equilibrium reaction as shown in Fig. 5, by the fact that at any pH between pH 8.5–10.5 nearly the same amount of native or denatured pepsinogen is obtained regardless of whether that pH is arrived at by raising the pH

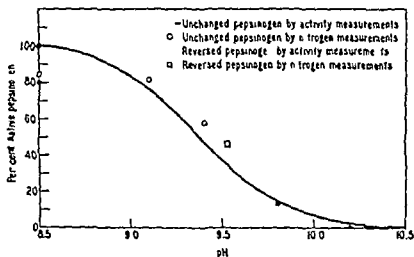
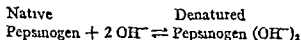


FIG. 5 Effect of pH on the equilibrium between native and denatured pepsinogen at 25°C.<sup>1</sup>

of a native activatable pepsinogen solution or by lowering the pH of a denatured unactivatable solution. The criteria of alkali denaturation, as in the case of reversible heat denaturation, is the insolubility in certain salt solutions in which native pepsinogen is

<sup>1</sup> If the logarithm of the ratio of native to denatured pepsinogen is plotted against pH, a straight line with a slope of 2 is obtained. This agrees with the assumption that the equilibrium between native and denatured pepsinogen is expressed by the following reaction—



or

$$\log \frac{[\text{Pepsinogen}]}{[\text{Pepsinogen (OH}^-\text{)}_2]} = 2 \log [\text{OH}^-] + \log K$$

soluble and the fact that denatured pepsinogen does not change into pepsin upon acidification. It may be seen that pH 8.5 is the best pH to reverse the alkali denatured pepsinogen.

### *Experimental Procedure*

*Pepsinogen Solution*—10 mg protein nitrogen per ml, pH 6.5 dialyzed salt free

*pH*—Determined by hydrogen electrode on mixtures of pepsinogen and the buffer. Electrode standardized in this region against Sørensen glycine and borate buffers.

*Procedure, I*—Approaching from the native side of the equilibrium, two 0.5 ml aliquots of the pepsinogen solution were added to two 4.5 ml aliquots of M/10 borate buffer of various pH values. After standing 5 minutes at 25°C, 5 ml of a 2 M magnesium sulfate in hydrochloric acid and 0.4 M pH 4.65 acetate was added to one aliquot and 5 ml of N/5 hydrochloric acid was added to the other. The hydrochloric acid in the salt solution was of such a concentration as to just neutralize the borate buffer. The protein salt solution was filtered and the nitrogen determined in the clear filtrate. Activity estimations by the hemoglobin method were made on N/5 hydrochloric acid-protein solution.

*II*—Approaching from the denatured side, 0.5 ml aliquots were mixed with 4.5 ml aliquots of M/10 pH 11.0 borate buffer, the final pH being 10.5, were allowed to stand 1 minute at 25°C which was found to be long enough to completely denature pepsinogen. They were then mixed with 5 ml of hydrochloric acid of just sufficient strength to change the pH to a desired value (pH 9.4 or 8.5) where the solutions were allowed to remain for 4 minutes. At this time, one aliquot of pepsinogen was mixed with 10 ml 2 M magnesium sulfate in 0.4 M pH 4.65 acetate while the other was mixed with 10 ml N/5 hydrochloric acid. These aliquots were then analyzed for nitrogen and activity as in *I*, the difference in dilution being considered.

The alkali denaturation of pepsin is almost an irreversible reaction when the protein concentration is comparable to that used in the pepsinogen experiments. A careful study by Steinhardt (4) brings out that the alkaline inactivation of pepsin is a first order reaction the rate of which is a function of the ionization of five titratable groups. He has suggested that the amino groups of cystine in pepsin may be involved. This seems unlikely to the writer who found that acetylation of the amino groups of pepsin had no detectable effect on the alkaline inactivation of the acetylated enzyme (13).

### *Secondary Alkali Change*

Between pH 8.5 and 10.5 practically complete reversal of denaturation can be effected if the exposure to the alkali is for a short time.

As the time of standing increases the extent of the reversal decreases. Fig. 6 shows the results of an experiment in which a solution of pepsinogen stood at pH 10.5 and 25°C for varying lengths of time after which reversal of denatured pepsinogen was allowed to take place for 30 minutes and the extent of reversal then determined. It may be seen from Fig. 6 that the denatured pepsinogen slowly changes into an irreversibly denatured form.

### Experimental Procedure

0.5 ml of a dialyzed solution of pepsinogen at pH 7.0 containing 10 mg protein nitrogen per ml was added to 4.5 ml of M/10 pH 11.0 borate buffer bringing the pH to 10.5. At intervals of time 1 ml of this solution was added to 1 ml of 0.075 N hydrochloric acid which changed the solution to pH 8.5. This was allowed to

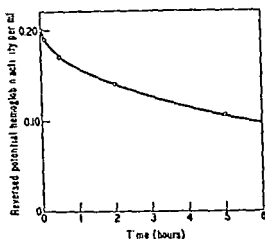


FIG. 6. Rate of change of reversibly denatured pepsinogen into an irreversible form at pH 10.5 and 25°C.

TABLE VII  
Amino Nitrogen as Per Cent of Total Nitrogen

Material	Formol titration	Van Slyke gasometric
Pepsin Native	1.4	1.4
Denatured	1.4	1.4
Pepsinogen Native	4.4	3.5
Denatured	4.2	2.5

stand at 25°C for 15 minutes which is sufficient to completely reverse the alkaline denaturation. After this 2 ml of M/10 hydrochloric acid was added and 10 minutes allowed for complete conversion of pepsinogen into pepsin after which it was diluted and the activity estimated by the hemoglobin method.

*Amino Nitrogen*

Table VII contains the amino nitrogen analysis of pepsinogen with pepsin as a control. Apparently the pepsinogen reacts with nitrous acid before it becomes activated since the value by the Van Slyke gasometric nitrous acid method (14) is not more than that by the formol titration, the latter method being carried out above pH 7.0. Pepsin contains less amino nitrogen than pepsinogen. This difference will be discussed in more detail later but it may be said here that part of the pepsinogen containing a relatively large amount of amino nitrogen is split off from pepsinogen upon its conversion into pepsin.

*Experimental Procedure*

The pepsin was dialyzed 24 hours at 10°C, first on the acid side of the isoelectric point against 0.02 N hydrochloric acid and then titrated to pH 4.5 and dialyzed 24 hours against  $\text{M}/1000$  pH 4.65 acetate buffer. The pepsinogen was dialyzed 24 hours at 10°C against  $\text{M}/1000$  pH 6.8 phosphate buffer, then solid potassium sulfate was added to bring the solution to approximately 0.5 M. This was then dialyzed 24 hours at 10°C against  $\text{M}/1000$  pH 6.8 phosphate. These procedures were designed to remove or displace all split products and ammonium ion bound as the salt of the protein.

The formol titration was carried out by the procedure described by Northrop (15). 50–150 mg of protein were used for each titration.

Denaturation was carried out by precipitating 50–150 mg of protein with 5–10 volumes of the following reagents: 2.5 and 10 per cent trichloroacetic acid, and by 0.5 M potassium sulfate. The protein was filtered or centrifuged and dissolved in dilute alkali. After solution of the precipitate the titration was carried out as usual except the solutions in which too much alkali was used to dissolve the precipitate, acid was added to bring them to pH 7.0.

In the Van Slyke gasometric procedure (14) the same amount of protein was used and denaturation was the same as in the formol titration experiments. After mixing the protein with the nitrous acid the chamber was shaken for 2 minutes, allowed to remain quiet for 23 minutes, and followed by a final shaking of 5 minutes.

*Isoelectric Point*

The isoelectric point of pepsinogen was determined by cataphoresis measurements (16) of pepsinogen coated collodion particles suspended in  $\text{M}/10$  acetate buffers of various pH values. By this method an isoelectric point of 3.7 was obtained. Reproducible values of unactivated pepsinogen were obtained by working rapidly with dilute solutions of pepsinogen although activation takes place at this pH.

The isoelectric point was also determined by finding the pH at which either the minimal amount of alcohol precipitated pepsinogen or the maximum precipitate was obtained with a constant amount of alcohol. This work had to be carried out at 0°C and as rapidly as possible to obtain a reading before appreciable activation took place. The breadth of the isoelectric region by these methods is certainly between pH 3.6 and 4.3 with 3.9 as the most likely isoelectric point. The isoelectric point of pepsin is pH 2.7 (5).

The pH measurements were made with a quinhydrone electrode standardized against known solutions of buffers.

### *Titration Curves*

Complete titration curves of pepsin and pepsinogen might lead to a correlation of certain titratable groups with particular properties of these proteins. Such correlation would be of greatest interest in the region of the titration curve from pH 1.0–5.0 where pepsin is catalytically active and pepsinogen is inactive. In this range, however, the titration curves could not be obtained with the degree of precision necessary for such a correlation.

On the other hand, the titration curves of these two proteins could be determined at pH more alkaline than pH 4.0 and the differences noted correlated with certain properties, namely the stability of instability at given alkalinities. In Fig. 7 are the curves showing the amount of alkali combined per gram of protein.

### *Experimental Procedure*

#### *Solutions*

*Pepsin*—2 times crystallized, dialyzed against M/1000 pH 4.65 acetate buffer and against 0.02 N hydrochloric acid followed by just enough alkali to dissolve. 7.5 mg total nitrogen per ml, 0.4 mg non protein nitrogen per ml,  $[P/U]_{mg}^{Hb} P.N. = 0.25$

*Pepsinogen*—Dialyzed, brought to 0.5 M with solid potassium sulfate and dialyzed 24 hours to remove this salt and any ammonium ion. 6.3 mg total nitrogen per ml, 0.0 mg non protein nitrogen per ml.

*Procedures*—50 ml of the pepsin plus 1 ml of 5.0 N sodium chloride were introduced into the titrating chamber at 25°C and the titration carried out with 5.0 N sodium hydroxide led into the solution from the capillary outlet of a microburette. The capillary tubing extended below the surface of the solution.

50 ml of the pepsinogen solution plus 1 ml of 5.0 N sodium chloride were cooled to 10°C and mixed rapidly with 0.13 ml. of 5.0 N hydrochloric acid. This was

done to obtain the minimum activation and yet the lowest pH on the activation curve. Less than 5 per cent activation occurred. The pepsinogen solution was then warmed to 25°C and poured into the titrating chamber and titrated with the same technique and alkali as was the pepsin. The electrodes were checked against  $M/10$  pH 4.65 acetate buffer and read 0.515 volt. The solutions were made  $M/10$  with respect to sodium chloride in order to raise the conductivity of the dialyzed protein solutions as well as to have the variation in ionic strength minimized during the experiment.

*Apparatus*—The writer has found it convenient to run the titrations in an enlarged modified Clark rocking hydrogen electrode chamber. The chamber of 125 ml capacity accommodates 50 ml of solution nicely. The hydrogen passes over the solution and saturates it as it rocks without any appreciable foaming. The platinized electrode is in and out of the solution as the angle of the chamber changes. A fresh saturated potassium chloride contact is made through a three-way stop-cock stuffed with cotton to minimize diffusion. The arrangement is such that the saturated potassium chloride in the stop-cock is washed out in between each reading and replaced with fresh saturated potassium chloride.

*Water Correction or Blank*—The correction for the quantity of alkali necessary to bring an equal volume of water to the various pH was experimentally determined with an equal volume of  $M/10$  sodium chloride using the same alkali, apparatus, and technique as in the protein titrations. This avoids errors due to factors such as carbonate in the alkali, etc.

The pepsinogen titration curve II was started at the lowest pH at which reproducible values of pepsinogen could be obtained before activation proceeded to an appreciable extent. In the time required for the measurements of pepsinogen in the region of pH 4.4 there was less than 5 per cent activation. Curve I was started at the most acid pH at which the pepsin solution could be titrated without the protein precipitating from solution. Since the pH at which the titrations were started are not the isoelectric points of the two proteins, curves I and II of Fig. 7 are not titration curves in the ordinary sense of the term. However, they do serve to show differences in the amount of alkali bound to the two proteins at various pH which in turn may be interpreted as meaning that there are more groups being titrated at a given pH in one protein than in the other.

Curve III is curve I plotted so that the value at pH 6.0 is the same as the pH 6.0 value of curve II. By so arranging them one can visually compare the slopes of the two curves at any pH.

It may be seen from curves II and III of Fig. 7 that between

pH 6.0 and pH 9.0 pepsin (curve III) binds 0.3 more millimols of alkali per gram of protein than does pepsinogen (curve II). In this region of alkalinity pepsinogen is unchanged, *i.e.* remains native, whereas pepsin is irreversibly denatured. Beyond pH 9.0 the pepsinogen curve rises sharply and approaches the pepsin curve at pH 12.0, indicating that pepsinogen has more groups titrating beyond pH 9.0 than does pepsin. Pepsinogen is also denatured by alkali but only beyond pH 9.0. This denaturation is, however, largely reversible (see section on Reversible alkaline denaturation). As may be seen in Fig. 7, curves II and III converge almost to a com-

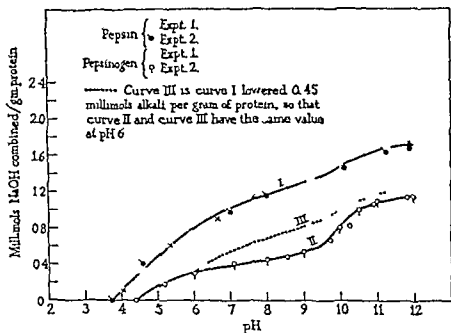


FIG. 7 Titration curves of pepsin and pepsinogen

mon point at pH 12.0. This means that the number of groups in pepsin and pepsinogen titrating between pH 6.0 and pH 12.0 is practically the same.

### *Molecular Weight*

There must be a difference in the molecular weights of pepsinogen and pepsin and the former must be the larger for, as will be explained more fully in a later section of this paper, conversion of pepsinogen into the active enzyme results in two substances—pepsin and a fragment the nitrogen of which is 15–20 per cent of the pepsinogen nitrogen.

*Experimental Procedure*

The protein solutions plus the amount of ammonium sulfate to bring them to the desired concentration were placed in collodion bags and connected to mercury filled manometers. A marble inside the membrane insured constant stirring. The membranes were surrounded by 400-500 ml. of the corresponding salt solution and the units rocked slowly in a thermostatically controlled room at 10°C. Each figure in Table VIII is the result of at least three readings taken at 12-24 hour intervals after the equilibrium was established. The protein estimation was made by Kjeldahl nitrogen determinations. The factor 7.0 was used to convert nitrogen analyses into amount of protein.

TABLE VIII

*Molecular Weight by Osmotic Pressure at 10°C in Two Concentrations of Ammonium Sulfate Solution.*

Material	Salt concentration	Osmotic pressure	Protein/ml.	Molecular weight
	<i>saturated</i>	<i>mm Hg</i>	<i>mg</i>	
Pepsin	0.15	22	48	39,000
	0.15	23	48	37,000
	0.15	26	47	32,000
	0.30	20	46	41,000
	0.30	22	52	42,000
				$\frac{38,000}{1.25} = 3,000$
Pepsinogen	0.15	15	36	42,500
	0.15	17	36	37,500
	0.15	16	34	37,500
	0.30	17	45	47,000
	0.30	19	45	42,500
	0.30	19	48	45,000
				$\frac{42,000}{1.4} = 3,000$

The figures in Table VIII reveal that the molecular weights calculated from osmotic pressure measurements bear out the above conclusion. The precision of the osmotic pressure measurements is such that one cannot be certain about the actual difference in the molecular weights. However, the difference is in the right direction and of the right order of magnitude.

*Tyrosine-Tryptophane Content*

In view of the writer's previous work (13, 17, 18) on the relationship of tyrosine of pepsin to its enzymatic activity it seemed worth while



to see if the tyrosine of pepsinogen differed in content or reactivity from that in pepsin. When an estimation of the content was made with Folin's phenol reagent and the color allowed to develop at pH 8.0 there was a decided difference in the values of pepsin and of pepsinogen, as shown in Table IX. It has been pointed out by Mirsky and Anson (19) that the tyrosine phenol groups of denatured proteins react more readily with reducing agents than when the protein is native. In view of the fact that pepsinogen is native at pH 8.0 whereas pepsin is denatured, it was necessary to determine whether the difference in the amounts of these amino acids was due to a difference in the reactivity of the tyrosine, or to the fact that the conditions were not comparable, i.e., the proteins were not both native or both denatured while being measured.

### *Experimental Procedure*

#### *Conditions for Color Development*

**pH 8.0**—The solutions, reagents, concentrations, etc., are similar to those previously described (17). One difference exists, namely that the 5 ml. of alkali phosphate solution was mixed with the 3 ml. of Folin's phenol reagent before they were added to the protein solution. If the acid phenol reagent were added to the protein solution before the alkaline solution was added activation might have occurred in the case of pepsinogen. On the other hand, if the alkaline solution were added first it would have denatured the pepsinogen. By mixing them together and then introducing them into the protein solution the pH never got above or below  $\text{pH } 8.0 \pm 0.2$ . The mixing must be rapid and even then occasionally a precipitate developed in the phenol reagent alkaline phosphate mixture. When this occurred the sample was discarded for only clear solutions should be compared in a colorimeter.

**pH 11.0–12.0**—To 19 ml. of a solution containing 1.5–2.0 mg. of protein was added 3 ml. of a one to three dilution of Folin's phenol reagent, followed by 3 ml. of 1.25 N sodium hydroxide solution. The solution at room temperature was read after 5 minutes against a similarly treated 0.15 mg. of tyrosine.

The results of the experiments on this point are given in Table IX. When the color reagent was applied in strong alkali, i.e. pH 11.0–12.0 or at pH 8.0 and  $70^{\circ}\text{C}$ , where pepsinogen is denatured, the values of pepsin and pepsinogen were nearly the same, showing that there is not an appreciable difference in the amount of the color giving amino acids present in the proteins but that the native and de

natured forms of pepsinogen behave differently in their action with the phenol reagent. It has not been found possible to determine the reactivity of tyrosine in native pepsin and a comparison of the native proteins is therefore impossible from this point of view.

### *Optical Rotation*

From the figures in Table X it appears that pepsinogen has a lower specific optical rotation than does pepsin. It also shows that crystal-

TABLE IX

*Tyrosine-Tryptophane Color Value (Expressed as Per Cent Tyrosine)*

Conditions for color development		Pepsin	Pepsinogen (1)	Pepsinogen (2)
pH	Temperature			
	°C			
8.0	35	5.0	3.2	3.2
8.0	70	5.6	5.1	5.5
11.0-12.0	25	9.3	8.8*	8.6*

\* The explanation for the difference between these values and those at pH 8.0 has already been discussed (17)

TABLE X

*Specific Optical Rotation of Crystalline Pepsins and Pepsinogens*

Material	$[\alpha]_D^{25^\circ C}$ gm dry weight
Crystalline Parke Davis pepsin	-72
Crystalline Cudahy pepsin	-71
Crystalline pepsin from pepsinogen	-72
Uncrystallized pepsinogen	-61
3 times crystallized pepsinogen	-62

line pepsin from pepsinogen has the same optical rotation as crystalline pepsin prepared from the commercial products by the methods previously outlined (5)

### *Carbohydrate*

The carbohydrate present in purified pepsin and pepsinogen preparations is less than 0.1 per cent which is less than 0.2 mol of hexose per mol of protein. It is therefore not to be considered as an intrinsic

part of the protein molecule. The methods used were essentially those of Sørensen and Haugaard (20) and of Elson and Morgan (21) the modification of the former being described under Experimental methods.

### *Ultraviolet Absorption Spectrum*

Although the plotted curves of the ultraviolet absorption spectra of pepsin and pepsinogen are indistinguishable, nevertheless a consistent difference exists which may be recognized on the plates. One of the tyrosine bands in pepsinogen is different from that of pepsin. The writer is indebted to Dr. George I. Lavin for these measurements.

### *Serology*

A detailed account of this subject has been given by Seastone and Herrriott (3).

### *Conversion of Pepsinogen into Pepsin*

When solutions of pepsinogen are made more acid than pH 6.0 the protein is converted into pepsin. This conversion has been studied from two different angles.

*Kinetics of Conversion*—As stated in the preliminary report (22), the conversion of pepsinogen into pepsin is an autocatalytic reaction at pH 4.6. Fig. 8 shows graphically the analyses of a conversion experiment in which the activity was determined directly by the gelatin viscosity method  $[P, U]^{Gel}$  and indirectly by the hemoglobin method  $[P, U]^{Hb}$ . The hemoglobin method as carried out measures the pepsinogen from which pepsin value is obtained by difference. It may be seen from Fig. 8 that the values by both methods fall reasonably close to the theoretical curve calculated from the average value of  $K$  using the simple autocatalytic formula

$$-\frac{dA}{dt} = KA(A - A_0)$$

which after integration gives

$$K = \frac{2.3}{At} \log \left( \frac{A}{A - 1} \right) \left( \frac{A - A_0}{A_0} \right)$$

This equation also describes the conversion of trypsinogen into trypsin (23)  $A$  is the activity at time  $t$ ,  $A_e$  is the final or equilibrium activity, and  $A_0$  is the initial value of  $A$  or activity at zero time

### Experimental Procedure

To 35 ml of a dialyzed solution of 3 times crystallized pepsinogen at pH 7.0 containing 1.4 mg protein nitrogen per ml, was added 13.0 ml of M/1 pH 4.5 acetate buffer. The entire system was kept at 25°C.

**Hemoglobin Activity**—0.5 ml aliquots of the activation mixture were added to 0.5 ml of saturated (0.3 N) borax making the solution pH 8.0. This inactivates the pepsin present. After 5 minutes these solutions were acidified to pH 1.0–2.0 where they were allowed to activate completely for 10 minutes. Activity was then determined in the usual way by the hemoglobin method.

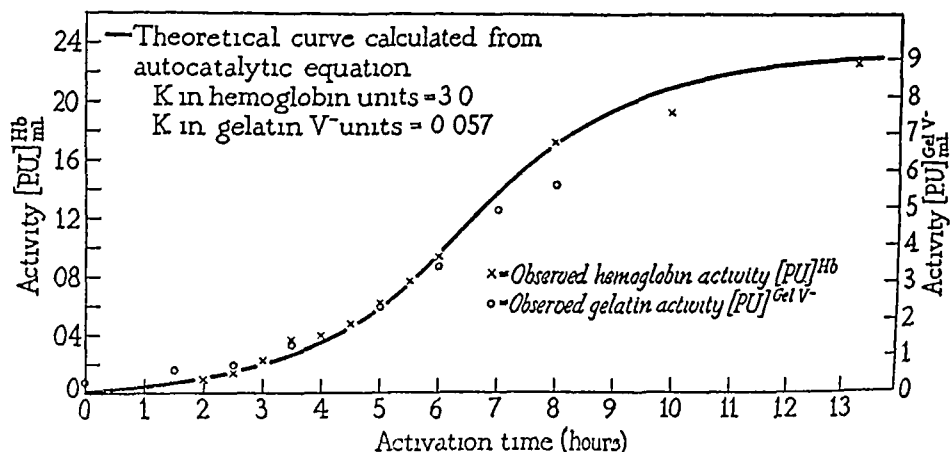


FIG. 8. Conversion of 1.0 mg pepsinogen nitrogen per ml into pepsin at pH 4.6 and 25°C.

**Gelatin Activity**—The gelatin activity was determined directly on aliquots of the activation mixture by measuring the change in viscosity of an isoelectric (pH 4.7) gelatin solution as described by Northrop (12).

The autocatalytic constant  $K$  for the experiment in Fig. 8 in hemoglobin activity units is  $3.0 \pm 0.1$ , *i.e.* when 1.0 mg of pepsinogen nitrogen per ml is activated at pH 4.6 at 25°C with a unit time as 1 hour and  $A$  expressed in hemoglobin activity units the fractional increase in  $A$  is 3.0 times per hour.

The conversion reaction rate varies enormously with pH. It is hardly perceptible at pH 6.0 while at pH 4.0 it is relatively rapid, *i.e.*, 1 mg protein nitrogen per ml at 25°C activates in about 30

minutes. Near pH 1.0 where the rate is at a maximum it is of the order of 100 times the rate at pH 4.0. In solutions more acid than pH 4.0 the kinetics of activation appear to be not the simple autocatalytic reaction noted between pH 4.5–5.0 though it can be demonstrated that it is at least partially autocatalytic as far acid as pH 0.0. This is shown by an increase in conversion rate by the addition of pepsin.

Activation rates are greatly increased by the presence of salts. Kunitz and Northrop noted a similar increase by salt in the activation of trypsinogen (8). The increase in the rate of conversion of pepsinogen by salt seems to be a function of charge or valency of the ions of the salt as well as the concentration of the salt. A shift in pH by the salt explains part of the increased rate of activation but there is still a definite effect which appears to be related to the presence of the salt at least in the case of magnesium sulfate at pH 4.0.

*Chemistry of Conversion*—When pepsinogen is changed into pepsin there is a concomitant production of non protein nitrogen (nitrogen not precipitable by 2.5 per cent trichloroacetic acid) to the extent of 15–20 per cent of the pepsinogen nitrogen. This is shown clearly in Fig. 9. Although the conversion was carried out under a number of different conditions of pH and temperature of which only two are shown the points all fall close to the same line in Fig. 9. That the line is straight in such a differential plot as A in Fig. 9 for two different sets of conditions and that the increase in non protein nitrogen stops at the same time that the increase in activity stops, as shown in B of Fig. 9, seems to exclude the possibility of this phenomenon being a coincidental digestion of a small amount of protein impurity. It cannot be supposed that the increase in non protein nitrogen is the complete digestion of part of the pepsinogen protein by pepsin since, as is shown in Table XI, the same amount of non protein nitrogen is produced from a given amount of pepsinogen even though three times the quantity of pepsin is added at the start.

### *Experimental Procedure*

*pH 2.0 Experiment*—To 150 ml. of dialyzed pepsinogen at pH 6.8 containing 5.5 mg. protein nitrogen per ml. cooled to 0°C. was added 140 ml. N/10 hydrochloric acid and 110 ml. water both of which had been cooled. At definite intervals of time 4 ml. aliquots of this solution were added to 10 ml. of 0.3 N

borax bringing the pH to 8.5. After standing 5 minutes at room temperature 2.0 ml aliquots of this alkaline solution were added to 10.0 ml of boiling 2.5 per cent trichloroacetic acid, cooled, filtered, and the nitrogen determined in the filtrate. The values in Fig. 9 were corrected to the original conditions of the reaction mixture. Another aliquot of the alkaline solution was added to hydrochloric acid of such concentration to bring the pH to 1.0–2.0 as indicated by thymol blue. Complete activation occurred within 5 minutes at room temperature. Activities were determined by the hemoglobin method. The value for 100 per cent activation was obtained by allowing an aliquot of the activation mixture to activate for 10 minutes at room temperature without going through the alkaline borax treatment.

*pH 4.7 Experiment*—To 15.0 ml of the same dialyzed pepsinogen solution was added 2.25 ml of  $N/10$  hydrochloric acid and 22.75 ml of  $M/10$  pH 4.65 acetate buffer. The solution was placed at 35.5°C and at definite intervals of time 4.0

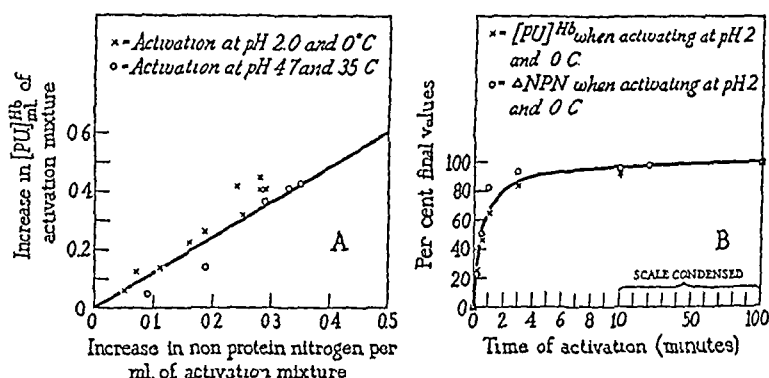


FIG. 9 Increase in non-protein nitrogen during activation at pH 2.0 and 4.7  
 A Increase in non-protein nitrogen with increased activation B Rate of increase in non-protein nitrogen and hemoglobin activity

ml aliquots were removed and treated in an identical manner as in the above pH 2.0 experiment

### Experimental Procedure

#### Protein Solutions

A—Dialyzed 2 times crystallized Cudahy pepsin, pH 3.5, 10 mg protein nitrogen per ml

B—Dialyzed 3 times crystallized pepsinogen, pH 7.0, 3.5 mg protein nitrogen per ml

First section of Table IX 5.0 ml A + 5.0 ml B

Second " " " " 5.0 ml B + 5.0 ml  $M/10$  pH 4.0 acetate buffer

Third " " " " 5.0 ml A + 5.0 ml  $M/10$  pH 4.3 " "

The final pH was practically 4.3 in all three sections. The solutions were kept at 35.5°C and 2 ml samples were added to 1 ml of 0.3  $N$  borax, bringing the pH

to 8.5 where it remained 5 minutes. 2.5 ml of these alkaline solutions was then precipitated with 10 ml of boiling 10 per cent trichloroacetic acid and the filtrate analyzed for nitrogen. The remaining 0.5 ml of alkaline mixture was activated with N/10 hydrochloric acid and analyzed by the hemoglobin method. The alkaline borax instantly inactivates the pepsin formed. Subsequent activation and analysis is a measure of the pepsinogen in the original activation sample.

Amino nitrogen analyses by the Van Slyke method show that upon conversion of pepsinogen to pepsin there is a total increase in amino nitrogen of not more than 0.3 per cent of the protein or 2.0 per cent of the total nitrogen which is equivalent to nine amino groups per

TABLE XI

*Effect of Added Pepsin on the Increase in Non Protein Nitrogen during the Activation of Pepsinogen*

Materials	Activation time	Extent of activation	Increase in non protein nitrogen
	min.	per cent	as 1/2 ml reaction mixture
Pepsin plus pepsinogen pH 4.3	0.33	37	0.11
	0.67	47	0.25
	1.00	61	0.21
	3.00	86	0.39
	420.00	—	0.63
Pepsinogen pH 4.3	2.00	35	0.18
	6.00	53	0.32
	15.00	84	0.41
	45.00	97	0.64
	420.00	—	0.52
Pepsin	420.00		0.00

molecule of protein. It is not known with certainty that the rupture of all nine of the linkages from which the amino groups were derived are involved or are necessary in the conversion.

Part, if not all, of the 15–20 per cent of the non protein nitrogen after being produced as a result of conversion of pepsinogen into pepsin combines with pepsin between pH 5.0–6.0 to form a dissociable inhibitor pepsin complex. That it inhibits pepsin is readily demonstrated by the rennet method which is carried out at pH 5.8. The inhibitor pepsin complex dissociates upon dilution and upon acidification. It does not combine with pepsin in acid solution so that it

does not affect the hemoglobin activity measurement but does eliminate the rennet method under certain conditions

### *Experimental Methods*

*Preparation of Copper Hydroxide Suspension*—2,500 gm of Merck's technical powdered copper sulfate is dissolved in 20 liters of tap water in a 50 liter battery jar. Into this solution, which is stirred mechanically, is run a solution made by mixing 800 ml of saturated (19.0 N) USP sodium hydroxide and 15–20 liters of water. The suspension is thoroughly stirred and the precipitate then allowed to settle for 12–24 hours after which time the supernatant liquor is decanted away. The jar is again filled with tap water, stirred, the precipitate again allowed to settle, and the supernatant liquor discarded. The precipitate is now stirred up with 40 liters of distilled water and allowed to settle. After decanting off the clear water this precipitate is referred to as washed copper hydroxide. After the last decantation the precipitate is usually the correct concentration for use. Its exact concentration may readily be determined by treating an aliquot with enough sulfuric acid to dissolve it and comparing the resulting solution in a colorimeter with a solution of copper sulfate of known concentration. Several such analyses have shown the washed copper hydroxide to be about 1 molar in concentration.

#### *Activity Measurements*

*A Estimation of Pepsin*—The solution is diluted and the activity determined as indicated below.

*B Estimation of Pepsinogen or Pepsinogen Plus Pepsin*—Acidify to pH 1.0–2.0, leave for 5 minutes at room temperature, dilute, and determine peptic activity.

*C Estimation of Pepsinogen Only in the Presence of Pepsin*—Titrate or bring to pH 8.5 and leave for 5–10 minutes, acidify to pH 1.0–2.0 for 5 minutes at room temperature, dilute, and determine peptic activity.

*D Estimation of Pepsin Only in the Presence of Pepsinogen*—This can be carried out by measuring the peptic activity directly by the rennet method or by the difference in the hemoglobin activity of two aliquots treated as in B and C.

*Hemoglobin*—This is the method of Anson and Mirsky (24).

*Rennet*—The writer has combined several of the desirable features of the rennet methods of this and other laboratories into the following simple, rapid, and easily reproducible method.

*"Klim" Solution*—20 gm of Klim are worked into a paste in a mortar with water, poured into a 100 ml graduate, and 10 ml of  $\times/1$  pH 5.0 acetate buffer is added, finally diluting the suspension to 100 ml with water. This solution should be kept in the ice box when not in use and for precise work it should not be used after 5 days from the time it is made up. A control tube should not clot in 24 hours at 35°C.

*Procedure*—5.0 ml of the above Klim solution is brought to 35.5°C in a water bath and 0.5 ml. of the pepsin solution diluted in  $\times/10$  pH 5.0 acetate is added.



The pipette should be held about 1 inch above the Klm solution and the last drop blown out of the pipette. The test tube containing the digestion mixture is now twirled once or twice to mix the solutions and to wash down any enzyme solution which may be on the side of the tube. A stop-watch is started as the enzyme is added. The tube is now left in the bath until a minute or two before it should clot and then the tube is tipped and slowly rotated so that the worker can examine a thin film of the solution which thickens and coagulates in small particles just before clotting. The end point is arbitrary and therefore depends upon the worker. However, the variation in the end point determined by two workers is not great. The writer has chosen the first definite recognizable change in the film of milk as the end point. The stop-watch is, of course, stopped at the end point. Constant tipping or rotating of the tube has practically no effect on the clotting time.

*Enzyme Solutions*—0.5 or 1.0 ml of a solution of crystalline pepsin containing 0.003–0.0001 mg nitrogen per ml when added to 5 or 10 ml of a 20 per cent Klm solution at 35.5°C will clot it in 1–30 minutes. Over this range the time of clotting is inversely proportional to the pepsin concentration and does not vary with the time of standing in the acetate buffer. In activation mixtures of pepsinogen on the other hand, the ratio of dilution to clotting time is not constant as with crystalline pepsin but increases with dilution. At any given dilution the activity also decreases with time of standing in the acetate buffer. This latter effect is more striking when the activation mixture is diluted in M/10 pH 5.6 acetate instead of the M/10 pH 5.0 buffer. This and other evidence indicates the presence of inhibiting material which, although dissociated in acid thus not affecting the hemoglobin method, combines with pepsin at pH 5.0–6.0 and reduces its rennet action. The inhibiting material may be separated from pepsin and on mixing with purified pepsin will give effects similar to those noted for activation mixtures. Crystallization of pepsin from an activation mixture leaves the inhibiting material in the mother liquor. It has recently been found that replacement of M/10 pH 5.0 acetate buffer by M/1 pH 5.0 acetate as the solvent for the Klm solution will greatly diminish these inhibitor effects, also that the activation mixture should be diluted in water and the aliquot added to the Klm solution immediately.

*Rennet Activity Units* [ $P U$ ]<sup>Ren</sup>—The writer has, for convenience, arbitrarily defined 1 rennet unit as the amount of enzyme which under the above defined conditions will clot 11 ml of the enzyme Klm mixture in 1 minute. This rennet unit is not to be confused with the previously described one (12). The rennet activity of any solution is obtained then by dividing the dilution by the time in minutes required to clot the Klm. Thus, if 1.0 ml of an enzyme solution was diluted to 500 ml. and 1.0 ml was added to 10.0 ml of the 20 per cent Klm or 0.5 ml. to 5.0 with a clotting time of 5 minutes the original solution would contain 500/5 or 100 [ $P U$ ]<sub>ml</sub><sup>Ren</sup>. The rennet activity per milligram of protein nitrogen [ $P U$ ]<sub>mg P.N.</sub><sup>Ren</sup> for a number of different swine pepsin preparations is given in Table IV.

*Carbohydrate Estimation* —The method of estimating carbohydrate used in these experiments was a modification of the Sørensen-Haugaard method (20). It is simple and rapid though not so precise or specific as the Sørensen-Haugaard scheme. The recent adaptation of their method by Heidelberger and Kendall (25) probably gives slightly more precise results than the writer's method which is as follows:

1.0 ml. of a solution of carbohydrate which will yield a color comparable to 0.1 mg. of glucose is introduced into a 50 ml. Erlenmeyer flask along with 15 ml. of 21.5 N sulfuric acid and 2.0 ml. of a 2 per cent solution of orcinol in 7.5 N sulfuric acid. The flask is placed in a stirred water bath at  $80^{\circ}\text{C} \pm 1.0^{\circ}$  for 30 minutes after which it is cooled in cold water and read in an ordinary colorimeter against a similarly treated standard 0.1 mg. of glucose or against a solution of pepsinogen which had previously been standardized in terms of glucose. A green glass over the eye piece of the colorimeter aids in balancing the solutions. The color is stable for several hours and the writer has run a dozen or more samples at one time with ease and reproducibility.

*Nitrogen Estimations* —The technique of running the Kjeldahls was that previously reported (11).

Protein nitrogens in the presence of appreciable amounts of ammonium sulfate were made by precipitating the protein from an aliquot with 5–10 volumes of boiling 5 or 10 per cent trichloroacetic acid, cooling and filtering the precipitate, and washing it on the funnel with cold 2.5 per cent trichloroacetic acid until the washings were free of ammonium ion as indicated by a negative Nessler test. The precipitate was then washed into a Kjeldahl flask and the nitrogen estimated as usual. With crude solutions of pepsinogen or in the presence of appreciable quantities of gastric mucin the protein nitrogens were very erratic due to incomplete precipitation or the solubility of the denatured material.

*pH Estimation* —For the most part the pH determinations were colorimetric using the indicators recommended by Clark and Lubs (26). In certain cases hydrogen quinhydrone and glass electrodes were used but those cases are indicated in the procedures of the individual experiments. The values obtained by the use of indicators are the pH values of molar tenth buffers which give the same color.

#### SUMMARY

1. A method is described for the preparation of pepsinogen from swine gastric mucosae which consists of extraction and fractional precipitation with ammonium sulfate solutions followed by two precipitations with a copper hydroxide reagent under particular conditions. Crystallization as very thin needles takes place at  $10^{\circ}\text{C}$ , pH 5.0 and from 0.4 saturated ammonium sulfate solution containing 3–5 mg. protein nitrogen per milliliter.

2 Solubility measurements, fractional recrystallization, and fractionation experiments based on separation after partial heat or alkali denaturation and after partial reversal of heat or alkali denaturation failed to reveal the presence of any protein impurity

3 The properties of the enzymatically inactive pepsinogen were studied and compared with the properties of crystalline pepsin. The properties of pepsinogen which are similar to those of pepsin are molecular weight, absorption spectrum, tyrosine tryptophane content, and elementary analysis. The properties in which they differ are enzymatic activity, crystalline form, amino nitrogen, titration curve, pH stability range, specific optical rotation, isoelectric point, and the reversibility of heat or alkali denaturation.

4 Conversion of pepsinogen into pepsin at pH 4.6 was found to be autocatalytic, i.e., the pepsin formed catalyzes the reaction. Conversion of pepsinogen into pepsin is accompanied by the splitting off of a portion of the molecule containing 15-20 per cent of the pepsinogen nitrogen.

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# CALCULATIONS OF BIOELECTRIC POTENTIALS

## II THE CONCENTRATION POTENTIAL OF KCl IN NITELLA

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In earlier studies<sup>1</sup> the electrical behavior of *Nitella* conformed to the equations of Nernst and of Henderson but recently, in a different set of cells,<sup>2</sup> we have found some apparent exceptions

These exceptions are illustrated in Fig 1 The theoretical curve shows the approximate change in P.D. which KCl would produce if the values were due entirely to diffusion potential in the protoplasmic surface<sup>3</sup> and the mobility of  $K^+$  greatly exceeded that of  $Cl^-$  The values were calculated from the equation

$$\text{Change of P.D.} = 58 \frac{U - V}{U + V} \log \frac{C_1}{C_2}$$

where  $U$  and  $V$  are the mobilities of  $K^+$  and  $Cl^-$  respectively,  $C_1$  and  $C_2$  are the concentrations,<sup>4</sup> and  $V$  is taken as zero All these values relate to the non aqueous surface layer of the protoplasm

It is evident that the slopes of the observed curves do not surpass that of the theoretical curve except at certain concentrations Thus when 0.001 M KCl is substituted for 0.000316 M the curve rises abruptly so that its slope exceeds the theoretical

<sup>1</sup> Osterhout, W J V, *J Gen Physiol*, 1929-30, 13, 715

<sup>2</sup> The earlier cells (which came from a different locality and will be called Lot A to distinguish them from the present cells which will be called Lot B) showed a lower concentration effect of NaCl and much less inhibitory effect of calcium on the production of negativity by KCl This will be discussed elsewhere

<sup>3</sup> It is assumed that the concentration of KCl in the protoplasmic surface is directly proportional to that in the external solution

<sup>4</sup> Concentrations are employed for convenience in place of activities If this were not done the straight line would become somewhat curved

The reason for this abrupt rise is evident when we examine the photographic record shown in Fig 2

The observations were made on *Nitella flexilis*, Ag and were recorded photographically

Short period recording devices require for their operation either high voltage,

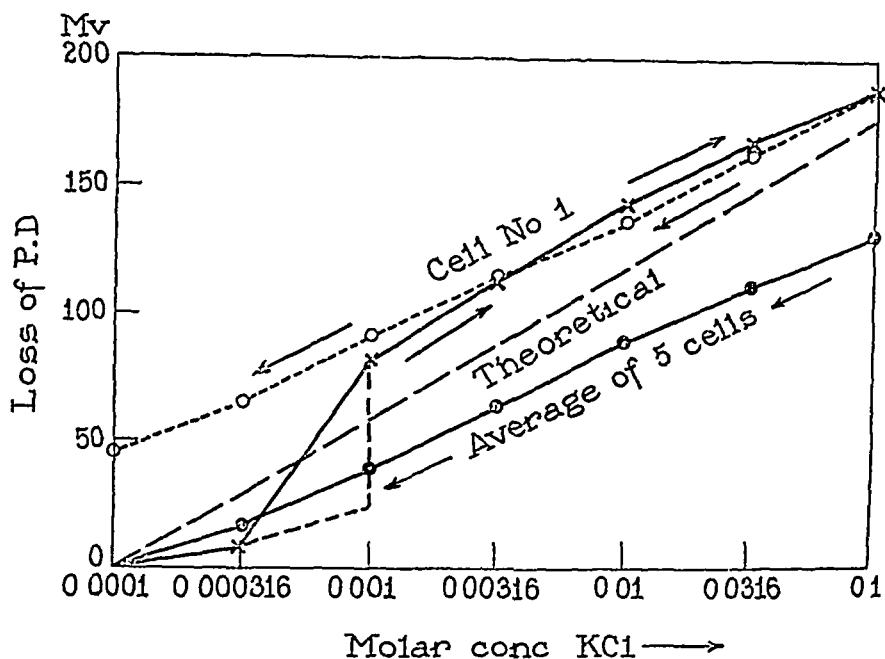


FIG 1 Effects of KCl on P.D. The broken straight line approximates the theoretical slope of the curve showing change of diffusion potential when the concentration of KCl in contact with *Nitella* increases and the mobility of  $K^+$  greatly exceeds that of  $Cl^-$ , partition coefficients being constant. The scale of abscissae is logarithmic each step is made by multiplying by 3.16 ( $= 10^{0.5}$ )

The curve with crosses shows measurements on a single cell as the concentration of KCl increases (arrows pointing upward). The curve with open circles (dotted line) shows measurements on the same cell as the concentration of KCl decreases (arrows pointing downward). The lowest curve shows the average of five cells as the concentration of KCl decreases (see p 556)

The slopes of the curves do not exceed the theoretical except in the curve with crosses where 0.000316 M KCl is replaced by 0.001 M KCl. At this point the change in P.D. occurs in two steps as indicated by the broken line. The first step does not exceed the theoretical, the second is larger and is due to an action current which permanently raises the level of the curve (see Fig 2)

Temperature 20–21°C

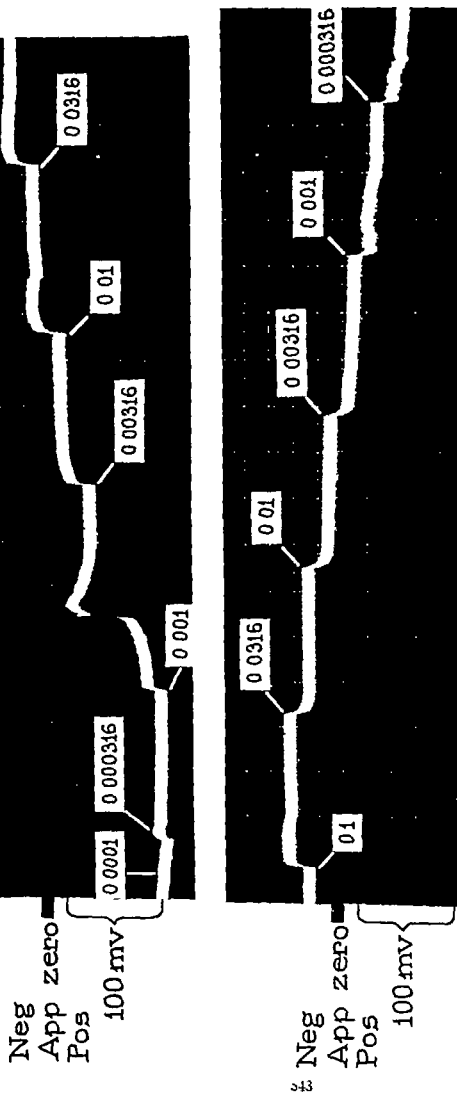


FIG. 2. Photographic record showing changes in p.d. produced by KCl. Three leads were arranged as shown in Fig. 3. The changes of solution were made only at D (the records of C and E which were in contact with pond water are omitted to save space). The p.d. at I (which was in contact with 0.01 M KCl) remained constant. It is assumed that its p.d. was approximately at zero as is usually the case and hence the label 'App zero' at the left (Cell No. 1 of Fig. 1).

At the start D was in contact with 0.0001 M KCl and (reckoning from the App zero) had a positive p.d. of 12 mV. When this was replaced by 0.000316 M KCl the curve rose 7 mV. When this was replaced by 0.001 M KCl the curve rose 15 mV and then an action current occurred which permanently raised the level of the curve so that on returning to 0.000316 M its level was higher than before (cf. Fig. 1). Heavy time marks 5 seconds apart.

large current, or both *Nitella* can furnish neither. A vacuum tube amplifier is therefore necessary.

The string galvanometer has adequate speed for *Nitella* and is used because of its simplicity. Tungsten wire replaces the quartz string, as a single tube amplifier is adequate and string breakage is eliminated. (The same amplifier may be used with a quartz string galvanometer by using a 20,000 ohm string shunt. Greater sensitivity and quicker period will result.)

The amplifier shown in Fig. 4 is designed for a galvanometer with tungsten wire in place of quartz fibre, and is grounded at *B*, the galvanometer wire being 90 volts above ground. The amplifier grounded at *B* should not be used with a quartz string unless the frame of the galvanometer is connected to the end

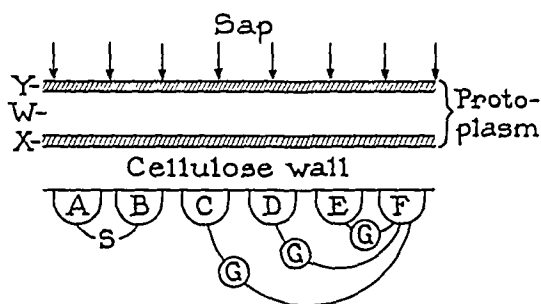


FIG. 3. Diagram to show the arrangement of leads and the supposed structure of the protoplasm which is assumed to consist of an aqueous layer *W*, an outer non-aqueous layer *X*, and an inner non-aqueous layer *Y*.

The arrows show the outwardly directed (positive) *P D* whose seat is supposed to be chiefly at *Y* when the cell is in pond water; hence the *P D* at *X* is regarded as negligible and is not shown. But under some conditions the *P D* at *X* may become important.

Each lead is connected to a separate amplifier and to one string of the 3 string Einthoven galvanometer.

of the string which goes to the slider of *P<sub>1</sub>*. If a high potential is applied between a quartz string and the frame of the instrument, the string will be attracted to the frame and the coating destroyed. This amplifier may be used with  $10^6$  ohms in the input circuit with little disturbance from *A C* lines, and if the *Nitella* cell and electrodes are placed in a shielded cage, its resistance may be as great as  $10^7$  ohms with little error. The vacuum tube is operated at its "free" grid potential in order to keep grid current at a minimum.

The function of the amplifier is to furnish current to the string galvanometer. With the circuit constants shown, for each volt change in grid potential there will be a change of 1500 microamperes in current through the galvanometer string. The linear range of the grid is about 0.25 volt each side of free grid potential. At the tungsten string tension employed, a change in grid potential of 0.05 volt



results in a string shadow movement of about 1 cm (0.2 meter per volt). The current flowing through the string ( $0.05 \times 1500 = 75$  microamperes) is about 400 times that necessary to produce a similar movement of a quartz string at conven

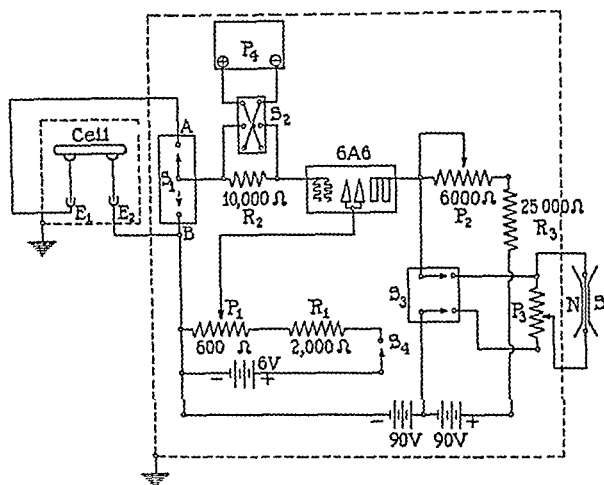


FIG 4 Arrangement of amplifier

$S_1 S_2 S_3$  = General Radio No 339 B low capacity switches

$S_1$  = toggle switch

$P_1 P_2$  = General Radio No 314 A potentiometers

$P_3$  = 600 ohm No 314 A General Radio potentiometer (for tungsten string)  
 = 20,000 ohm No 314 A General Radio potentiometer (for quartz string)

$P_4$  = any available shielded potentiometer

$R_1 R_2 R_3$  = International Resistance Co 10 watt wire wound resistors

$E_1 E_2$  = saturated calomel electrodes

Cell = single cell of *Nitella*

tional electrocardiogram tensions. Therefore the sensitivity with a quartz string instrument would be  $0.050 \div 400$  or  $0.000125$  volt per cm (80 meters per volt).

In use switch 1 is thrown to position 4 with no cell in the circuit and  $P_2$  adjusted until the galvanometer string is at zero.  $S_1$  is then thrown to position B,

and  $P_1$  is adjusted until the string is again at zero. This procedure is repeated once. With  $S_1$  in position  $B$ , a calibrating potential is now applied across  $R_2$ , and  $P_3$  is adjusted until the desired sensitivity is reached. Alternatively, the string tension may be adjusted. With the 3-string galvanometer, it is our custom to adjust the strings to approximately the same deflection with the same settings of the three potentiometers ( $P_3$ ) and then make exact adjustments with the potentiometers. With a cell in the circuit, the calibrating potential is recorded. No difference will be found between the series and direct calibrations if the amplifier is in proper adjustment.

The apparatus is assembled in grounded iron boxes of the sort obtainable at radio supply stores, and all external wires are covered with grounded copper shielding. All controls have insulated shafts extending through the shielding.

All measurements were made from photographic records.

The plants are transported directly from the pond to the laboratory and immediately washed in tap water with as little mechanical manipulation as possible. They are then placed in Solution A<sup>5</sup> in enamel ware tubs covered with glass plates and kept in a cold room at  $15^\circ \pm 1^\circ\text{C}$ .

To prepare a cell for experiments neighboring cells are cut away, leaving at each end a strip of dead cell wall about 10 mm in length by which the cell can be picked up with bone-tipped forceps which do not actually touch the living cell.<sup>6</sup> The cells thus prepared are allowed to stand for several days in Solution A before being used.

The experiments described in this paper were made with flowing contacts, as shown in Fig 5. A paraffin block  $P$  is shown in cross-section with a strip of filter paper  $F$  resting on it. The *Nitella* cell  $N$  rests on this and is covered with a thin layer of moist cotton  $C$ . Solution runs from the tube  $T$  over the cell and down to the cup  $B$ . The filter paper touches the tube  $T$  and the cup which in turn touches the waste beaker so that no drops are formed at any point. A continuous flow is maintained even during a change of solutions. For this purpose the old solution is allowed to run out until the funnel is nearly empty. The new solution is then poured in so that it follows the old solution without interruption.

Connections to the string galvanometer (through the calomel electrodes) are made as shown in Fig 3. Care is taken to maintain a moist atmosphere around the exposed parts of the cell.

The pH of the solutions of inorganic salts between 6 and 9 has little effect on *Nitella* and no especial precautions on this score are needed.

The temperature varied between 20 and 21°C.

In making the record shown in Fig 2, three places on the cell ( $C$ ,  $D$ , and  $E$ , Fig 3) were connected (through separate amplifiers and

<sup>5</sup> For the composition of this see Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933-34, 17, 87.

<sup>6</sup> Cf Osterhout, W J V, *Biol Rev*, 1931, 6, 369, *Ergebn Physiol*, 1933, 35,

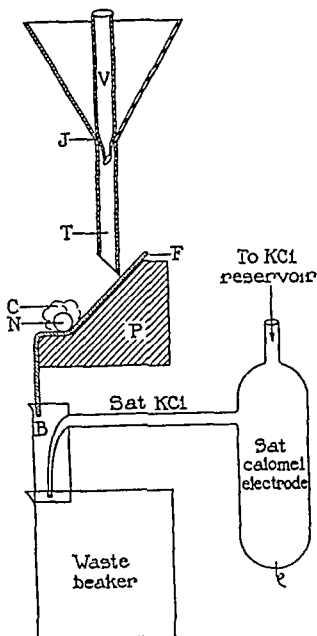


FIG 5 Shows a cross-section of the arrangement of flowing contact. The solution is poured into the funnel. The rate of flow is determined by a groove in the ground joint *J*. Formation of slugs of water in the 3 mm tube is prevented by the vent tube *V*. The solutions flow from the upright tube *T* to filter paper *F* which lies on a paraffin block *P*. On this rests a *Nitella* cell *N*, covered with moist cotton, *C*; thus the solution flows completely around the cell. The filter paper (several layers) touches the tube *T* and enters the cup *B* filled with saturated KCl. Cup *B* overflows into a waste beaker which it touches so that formation of drops is precluded.

Connection to the calomel electrode is made by means of a tube filled with saturated KCl; this tube is fused with cup *B* and with the calomel electrode vessel. The bridge and cup are flushed out by a constant small flow of saturated KCl from a reservoir.

The P.D. of the liquid junction between the saturated KCl in cup *B* and the other solution at the top of cup *B* is in most cases negligible.

through the 3-string Einthoven galvanometer) to a spot  $F$  at the right end of the cell. The spot  $F$  was in contact with 0.01 M KCl which kept the  $pD$  constant, approximately at zero.<sup>7</sup> Any change in  $pD$  at  $F$  would cause simultaneous changes at  $C$ ,  $D$ , and  $E$ . The absence of such changes was shown by the records<sup>8</sup> of  $C$  and  $E$  (omitted to save space). Hence we may be sure that all the alterations seen in Fig. 2 took place at  $D$ .

The record starts with 0.0001 M KCl at  $D$  which shows a positive<sup>9</sup> potential<sup>10</sup> of 125 mv. When the external concentration was raised to 0.000316 M the curve rose 7 mv (indicating a loss of potential<sup>11</sup>).

When 0.001 M KCl was applied the curve jumped up 15 mv. This was soon followed by a gradual rise and an action current<sup>12</sup> after which the level of the curve remained considerably higher.

This raises some interesting questions which involve the structure of the protoplasm. We suppose that the protoplasm consists of an aqueous layer  $W$  (Fig. 3, p. 544) with an outer ( $X$ ) and an inner ( $Y$ ) non-aqueous layer. The outwardly directed (positive)  $pD$  appears to be due to an outward gradient<sup>13</sup> of  $K^+$  across  $Y$ .

When the potential at  $D$  has been lowered by 22 mv (by applying 0.000316 M KCl followed by 0.001 M) we may suppose that an adjoining region  $D_1$ , only a few microns from the edge of the drop of 0.001 M KCl covering  $D_1$ , discharges into  $D$  in the usual way<sup>13,14</sup>. This, of

<sup>7</sup> This was not measured on this cell but was determined for other cells of the same lot by leading off from a spot in contact with 0.01 M KCl to one in contact with 0.01 M KCl saturated with chloroform which latter reduces the  $pD$  to zero.

<sup>8</sup>  $C$  and  $E$  were in contact with pond water during the entire experiment.

<sup>9</sup> The potential is regarded as positive when positive current tends to flow from the sap across the protoplasm to the external solution.

<sup>10</sup> This value is reckoned from the zero given on the record which depends on the assumption that the  $pD$  at  $F$  is zero (cf. footnote 7).

<sup>11</sup> See earlier experiments, Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 761; Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715.

<sup>12</sup> It might be thought that this comes from mechanical stimulation but in that case the start of the action current would be abrupt and not gradual (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930-31, 14, 473). Mechanical stimulation is very improbable in view of the precautions taken to avoid it in changing solutions (see p. 546).

<sup>13</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 215.

<sup>14</sup> Osterhout, W. J. V., *Biol. Rev.*, 1931, 6, 369.

course, is not recorded<sup>15</sup> at  $D$ . It may involve only a partial loss of  $P.D.$  at  $D_1$  for we find that it is not propagated to  $C$  and  $E$ <sup>16</sup> (this is often the case with discharges involving incomplete loss of potential in *Nitella*)

We suppose that such a discharge involves an increase in permeability at  $D_1$  accompanied by a movement of substances (organic and inorganic) from the sap into  $W$ . If these substances diffusing along  $W$  to  $D$  (only a few microns distant) cause an increase in the permeability<sup>17</sup> of  $Y$  at  $D$  we can understand why an action current occurs at  $D$ . The delay<sup>18</sup> after the application of 0.001 M KCl would be due to the time required for the diffusion of substances from  $D_1$  to  $D$  in  $W$ .

The loss of potential due to the action current at  $D$ , amounting to 77 mv. at the spike, is presumably larger than at  $D_1$ . At any rate it is propagated and appears at  $C$  and  $E$ .

When an ascending series of concentrations of KCl is applied an action current is regularly encountered at 0.001 M to 0.005 M KCl.

We suppose therefore that the action current at  $D$  is brought about by the application of KCl which depresses the  $P.D.$  at  $D$ . But such a depression brought about by the application of NaCl seldom produces an action current<sup>19</sup>. This may be due to the fact that, as Blinks has shown,<sup>20</sup> KCl lowers the resistance of the protoplasm much more than NaCl does. The lowered resistance would facilitate the discharge of  $D_1$  into  $D$ . It is possible that the presence of KCl in the external solution acts in other ways to facilitate the production of the action current.

<sup>15</sup> This is to be expected since there need be no change of  $P.D.$  at  $D$ . When a discharge occurs the change of  $P.D.$  takes place at the source and not at the sink. This is clearly shown when the sink is a dead spot.

<sup>16</sup> The records of  $C$  and  $E$  are omitted to save space.

<sup>17</sup> It may seem strange that  $Y$  which is in contact with sap at its inner surface should suffer an increase in permeability when sap reaches the outer surface. But this is less surprising when we remember that *Valonia* soon dies when placed in its own sap and that the process of death is accompanied by a great increase in permeability in both  $Y$  and  $X$ . Cf. Osterhout, W. J. V., *J. Gen. Physiol.* 1924-25, 7, 561.

<sup>18</sup> As would be expected the duration of this delay is variable.

<sup>19</sup> When an action current occurs there is no extra loss.

<sup>20</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 495.

When an action current is produced by KCl the subsequent level of the curve is higher than before. For convenience we shall refer to this as the "extra loss" of potential due to the action current. This extra loss is evident in the subsequent course of the curve even after the external KCl has been raised to 0.1 M and lowered again to 0.000316 M for we then find the curve at a higher level than when 0.000316 M KCl was first applied (Figs. 1 and 2).

In order to see whether the extra loss remains longer in evidence the external KCl was again raised to 0.1 M and lowered to 0.000316 M (stepwise as in Fig. 2). Above 0.001 M this curve practically duplicated that in Fig. 2 and the extra loss was in evidence throughout.

Is the extra loss due to changes in  $X$  or in  $Y$  or in both? Let us first discuss  $X$ . In previous experiments we have observed a permanent loss of potential after an action current. This has been explained as follows<sup>21</sup>. The spike of the action current is due to an increase in the permeability of  $Y$  which allows  $K^+$  to move out of the sap (where its concentration is about 0.05 M) into  $W$ . This lessens the gradient<sup>22</sup> of  $K^+$  across  $Y$  and hence lowers the outwardly directed (positive) potential. At the same time an organic substance, called for convenience<sup>23</sup>  $R$ , coming out of the sap makes  $X$  more sensitive to the action of  $K^+$  and thus increases the inwardly directed (negative) potential due to the external KCl acting on  $X$ . We suppose that even if  $Y$  regains its original positive potential during recovery there remains the extra loss of potential due to the increased effect on  $X$  of the external KCl.

If the increased sensitivity of  $X$  to KCl is due to an organic substance  $R$  which comes out of the cell sap we might expect the extra loss of P.D. to persist as long as  $R$  remains in  $X$ . It would seem that there is one group of substances, which may be called  $R_p$  for convenience, which increases the sensitivity of  $X$  to KCl and another group, which may be called  $R_a$ , which facilitates the production of action currents. There is some unpublished evidence<sup>24</sup> that potassium

<sup>21</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934-35, 18, 681.

<sup>22</sup> The loss of P.D. might also be due to mechanical breaks in  $Y$ . Cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, 11, 673. Hill, S. E., *J. Gen. Physiol.*, 1934-35, 18, 357. Osterhout, W. J. V., *Proc. Nat. Acad. Sci.*, 1938, 24, 75.

<sup>23</sup> Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 687.

<sup>24</sup> This will be discussed in another paper.

combines with a substance which we may call HZ to form KZ, which is identical with  $R_p$ . Hence we might expect that if  $K^+$  is removed from the external solution the  $R_p$  in  $X$  would tend to lose its potassium and consequently to lose its efficiency. This seems to be the case. The extra sensitivity to KCl and consequently the extra loss of P.D. gradually disappears when the external solution of KCl is replaced by pond water, or by Solution A or by a solution of NaCl.<sup>25</sup> It may even disappear in a few minutes in 0.0001 M KCl.<sup>6</sup> In higher concentrations of KCl its disappearance is much slower. Theoretically we might expect it to disappear eventually even in the higher concentrations of KCl since it would tend to diffuse out into the external solution.

How does this extra loss of P.D. come about? Let us return to the equation given on p. 541 which may be written

$$\text{Change of P.D.} = 58 \frac{U - V}{U + V} \log \frac{S C_1}{S C_2}$$

where  $C_1$  and  $C_2$  are the concentrations in the external solution and  $S$  is the partition coefficient (conc. in  $X$  - conc. in the external solution). If  $S$  remains approximately constant<sup>27</sup> the slope of the curves in Fig. 1 will depend on the value of  $(U - V) - (U + V)$ . Before the action current, when the concentration of KCl is raised from 0.000316 M to 0.001 M, we have (using concentrations for convenience in place of activities)

$$15 = 58 \frac{U - V}{U + V} \log \frac{S 0.001}{S 0.000316}$$

$$\frac{15}{58 (0.5)} = \frac{U - V}{U + V}$$

<sup>25</sup> In 0.01 M NaCl or in more dilute solutions it may disappear in less than a minute. The test is made by substituting NaCl for KCl and then replacing KCl of the same concentration as before to see whether the same P.D. is observed.

<sup>26</sup> This is most easily observed by treating the cell as in Fig. 2 and when the concentration has been lowered from 0.1 M to 0.0001 M KCl leaving it until the excess loss of potential gradually disappears, as shown by the gradual downward drift of the curve.

<sup>27</sup> This is probably true under normal conditions in the absence of action currents.

whence

$$(U - V) - (U + V) = 0.52$$

When the next change of concentration is made we have

$$28 = 58 \frac{U - V}{U + V} \log \frac{0.00316}{0.001}$$

$$\frac{28}{58 (0.5)} = \frac{U - V}{U + V}$$

whence  $(U - V) - (U + V) = 1$  (this can happen only when  $U$  is extremely large as compared to  $V$ )

Evidently therefore we cannot expect the change of P.D. to exceed<sup>23</sup> 28 mv no matter how much the value of  $U$  is increased by the action of  $R$  coming out of the sap for it cannot raise the value of  $U - V$  above unity

Apparently the action current causes  $R$  to come out of the sap and this raises the value of  $(U - V) - (U + V)$ , so that the change in P.D. is 28 mv instead of 15 mv. In other words the action of  $R$  adds  $28 - 15 = 13$  mv to the change of P.D. and this 13 mv appears as part of the extra loss of P.D. But as the total extra loss is 60 mv we still have  $60 - 13 = 47$  mv to account for. This extra 47 mv must be due to the action of  $R$  in raising  $S$  to  $S'$ . The amount of this rise can be calculated as follows. We may write

$$\text{Change of P.D. due to change of } S \text{ to } S' = 58 \log \frac{S'}{S}$$

When this change is 47 mv we have

$$47 = 58 \log \frac{S'}{S}$$

whence  $S' - S = 6.5$

On this basis it would appear that we can distinguish between changes in partition coefficients and changes in mobility. It may be noted that this is not possible with the equations ordinarily used for phase boundary potentials

<sup>23</sup> The value  $0.001 - 0.000316$  in the equation will be lessened when activities are employed



Great changes in partition coefficients may be caused by the addition of organic substances as has been repeatedly shown in unpublished experiments on models in this laboratory.<sup>9</sup>

After the action current there is a considerable increase in the potassium effect, *i.e.*, the loss of P.D. produced by substituting a given concentration of KCl for the same concentration of NaCl. Before the action current this amounts to from 15 to 25 mv. After the action current it is 45 to 65 mv. This was also observed in earlier experiments.

We suppose that this indicates a greater increase in the partition coefficient<sup>30</sup> of KCl than in that of NaCl after the action current for the concentration effect of NaCl showed little or no change which indicates that the mobility<sup>31</sup> of  $\text{Na}^+$  remained approximately constant. Hence the increase in the potassium effect must be due to changes in partition coefficient rather than in mobility. (For descending series see p. 555.)

It may be added that the experiment shown in Fig. 2 has been varied by placing the cell at first in 0.000316 M NaCl and then transferring to 0.000316 M KCl. In a typical experiment the curve jumped up 35 mv. when the KCl was applied. This was followed by a slow rise and an action current after which the level of the curve was 25 mv. higher than just before the action current. Thus the behavior of the curve before and after the action current resembled that in Fig. 2.

<sup>29</sup> The expression "partition coefficient" as here used should be interpreted in a very liberal sense to include such cases as the following. When 0.2 M  $\text{Ba}(\text{OH})_2$  in water is shaken with isoamyl alcohol the partition coefficient ( $\text{Ba}^{++}$  in amyl alcohol  $\div$   $\text{Ba}^{++}$  in water) is less than 0.0005, but when the amyl alcohol contains 0.1 M oleic acid the partition coefficient rises to 0.029 because barium oleate is formed. When the aqueous solution of  $\text{Ba}(\text{OH})_2$  is 0.0015 M the partition coefficient rises to 3.46 the corresponding figure for  $\text{Ca}(\text{OH})_2$  is about 16.8. Temperature about 22°C.

<sup>30</sup> In earlier experiments the increase in the potassium effect involved an increase of the mobility of  $\text{K}^+$  as shown by the increase in the slope of the curve of concentration potential (when plotted as in Fig. 1). Cf. Osterhout, W. J. V., *J. Gen. Physiol.* 1934-35 18, 987.

<sup>31</sup> In these cells (Lot B) the mobility of  $\text{Na}^+$  (as shown by the concentration effect) was much higher than in the cells studied earlier (Lot A).

This discussion indicates that we may account for the excess loss of potential by changes in  $X$ . But it is probable that changes occur in  $Y$  and it would seem natural to look to these to explain the fact that after the action current in Fig 2 no subsequent action currents are produced<sup>32</sup> by further increases<sup>33</sup> in the concentration of KCl. This may also be due, in part at least, to the fact that on standing the diffusion boundary between  $D$  and  $D_1$  becomes more diffuse and this makes more difficult a discharge from  $D_1$  into  $D$  (this has been discussed in a previous paper<sup>34</sup>).

It is also possible that changes in  $Y$  might account, in part at least, for the excess loss of potential, *e g* by changing partition coefficients or mobilities or by a mechanical alteration (*e g* producing a "leaky" condition)<sup>22</sup>.

All of these suggestions are put forward merely as working hypotheses which may serve to bring the facts under a common viewpoint. Future investigation must decide their actual value.

It seems desirable before leaving this subject to consider briefly the sources of these potentials. They are, of course, thermodynamic<sup>35</sup> as distinguished from zeta potentials. In previous studies the electrical behavior of the cell could be predicted by using the equations for diffusion potential<sup>36</sup> rather than those for phase boundary potential<sup>37</sup>. In consequence they have been regarded for convenience as diffusion potentials.

<sup>32</sup> In a few cases a second action current occurred at the next increase in the concentration of KCl. Perhaps in these cases the changes produced by the first action current were less complete.

<sup>33</sup> Even when all the steps shown in Fig 2 were immediately repeated on this cell no action current occurred.

<sup>34</sup> Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1929-30, 13, 547.

<sup>35</sup> This is shown by the fact that they can be measured by means of a galvanometer. According to L R Blinks (personal communication) *Halocystis Osterhoutii*, Blinks and Blinks, can produce continuously for several days a current about 2.5 microamperes per cm<sup>2</sup> of cell surface. For technique see Blinks, L R, *J Gen Physiol*, 1935-36, 19, 875.

<sup>36</sup> Osterhout, W J V, *J Gen Physiol*, 1929-30, 13, 715. Damon, E B, *J Gen Physiol*, 1932-33, 16, 375.

<sup>37</sup> Donnan potentials need not be considered since nothing resembling a Donnan equilibrium exists and oxidation-reduction potentials are ruled out because no metallic electrodes were in contact with the cell (*cf* Osterhout, W J V, and Hill, S E, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1936, 4, 43).

In the cells previously studied the rôle of  $K^+$  was so predominant that other ions were neglected. For convenience we shall continue to do this but it should be understood that  $Na^+$  plays a more important rôle in the present cells (Lot B) than in the earlier ones (Lot A). We know that potassium enters and reaches a much higher concentration in the sap than in the pond water.

One way in which P D might be brought about is seen in artificial cells<sup>38</sup> where the protoplasm is represented by guaiacol. When a dilute solution of potassium is placed outside the artificial cell potassium enters until its concentration in the artificial sap inside the cell is much higher than outside. During this process the potential due to the potassium compounds undergoes a change of sign. At first it is negative (inwardly directed) but as  $K^+$  accumulates in the artificial sap the sign of the potential becomes positive (outwardly directed) as in *Nitella*.

We suppose that the positive potential in *Nitella* is due chiefly to compounds of potassium and sodium which reach a higher concentration in the sap than in the external solution. This appears to be chiefly due to a concentration gradient across  $Y$ , i.e. to a gradient from the sap, which contains about 0.05 M KCl and 0.05 M NaCl, across  $Y$  to  $W$ , which appears to contain very little of either.

We may suppose that when the cell is in pond water there is not much potential across  $X$  but when sufficient KCl or NaCl is added to the external solution an inwardly directed potential appears at  $\lambda$ . We may make the usual assumption that the outermost portion of  $\lambda$  comes almost instantaneously into equilibrium with the external solution and that the innermost portion of  $\lambda$  likewise comes into equilibrium with  $W$ . During such experiments as are shown in Fig. 2 the penetration of KCl at one spot on the cell may not have much effect on  $W$  which is constantly stirred by protoplasmic movement.<sup>39</sup>

If the concentration of  $K^+$  in  $W$  remains constant and the external concentration of KCl is raised from 0.001 M to 0.1 M it should theoretically make no difference as far as P D is concerned whether we do this suddenly or stepwise (as in Fig. 2). In other words it makes no difference theoretically whether the diffusion boundary in  $W$  is sharp or diffuse.<sup>40</sup> Practically however, we do find differences in experiments on diffusion in aqueous solutions when more than one electrolyte is involved but they are irregular and not predictable.

When the external concentration is suddenly raised from 0.001 to 0.01 M we suppose that a sharp diffusion boundary is formed in  $\lambda$  as discussed in a previous paper.<sup>1</sup>

Let us now consider briefly the effect of starting with 0.1 M KCl and proceeding stepwise in a descending series. The application of 0.1 M

<sup>38</sup> Osterhout, W J V. *J Gen Physiol*, 1932-33, 16, 157.

<sup>39</sup>  $W$  extends the whole length of the cell (usually 10 cm. or more) and the spot  $D$  where KCl is applied is only a centimeter in width. The thickness of  $W$  is undoubtedly much greater than that of  $X$ .

<sup>40</sup> MacInnes, D. A. and Yeh, Y. L. *J Am Chem Soc*, 1921, 43, 2563. Brown, A. S. and MacInnes, D. A., *J Am Chem Soc*, 1935, 57, 1356.

KCl causes an action current as would be expected from what is said earlier (p 549) but there is no delay since the curve jumps up at once to beyond zero owing to the action of the external KCl on  $X$  plus the effect of the loss of  $P D$  at  $Y$  which is quickly superimposed<sup>41</sup> As in the ascending series there is a partial recovery after which the extra loss persists To avoid superimposition the curve in Fig 1 has been made to pass through the origin and hence does not show the extra loss

In this case we find that the potassium effect is greater in the concentrated than in the dilute solutions This may be due, in part at least, to contamination of the dilute solutions

The descending series of concentrations in Fig 1 (lowest curve showing the average of five cells) gives a smooth curve Since the slope of this curve approaches the theoretical limit<sup>42</sup> it is evident that the high value (85.45) for  $U_K - V_{Cl}$  given in a former paper is justified

These results clearly show the importance of using methods of measurement which allow us to detect action currents The information afforded by continuous records is often indispensable

#### SUMMARY

Cells of *Nitella* have been studied which behave differently from those described in earlier papers They show unexpectedly large changes in  $P D$  with certain concentrations of KCl This is due to the production of action currents (these are recorded at the spot where KCl is applied)

A method is given for the separate evaluation of changes of  $P D$  due to partition coefficients and those due to mobilities

A new amplifier and an improved flowing contact are described

<sup>41</sup> This effect on  $Y$  appears more quickly than in the action current of the ascending series This might be expected since 0.1 M KCl would lower the resistance of the protoplasm more than would lower concentrations

<sup>42</sup> The theoretical slope becomes a little less when activities are taken into account Cf footnote 1

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# THE EXCITABILITY OF FROG MUSCLE WITH PARTICULAR REFERENCE TO ITS LATENT ADDITION

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Muscle substance appears to possess distinct advantages over nerve for the study of the dependence of the process of electrical excitation on various reagents such as drugs and inorganic salts for not only is there more known about the disposition of such substances in muscle, but further knowledge is probably much more easily obtainable. Unfortunately, however, Keith Lucas' assignment of the  $\alpha$  excitability to the muscle substance has not been wholly acceptable and in particular, it has been questioned whether the  $\alpha$  strength duration curve is a "true" curve, in the sense of whether or not it is the simplest procurable. On the other hand, the direct stimulation of muscle with wire electrodes almost certainly may involve the nerve (if it does not always do so, as many believe) so its use probably yields more information about nerve than muscle.

Since Rushton's (1930) extensive work on the subject, there seems to be little doubt that the  $\alpha$  curve is of the muscle, but a decision on the other matter as to whether it is the least distorted by extraneous factors is more difficult to make, and there is probably no very general agreement on this question.

One method of attacking the problem is to assume that a given mathematical form for the strength duration curve, *e g* that derived from data on nerve, is the simplest and least distorted, so that it may be used as a criterion for the "trueness" of other curves. Lapicque (1931*a*) in fact, used his canonical equation (1926)<sup>1</sup> to show that the  $\alpha$  excitability is not of the usual class. This conclusion can only be tentative, however, even though the canon is adequate for one excit-

<sup>1</sup> Lapicque (1926) p 214

ability, because the "trueness" of the canon itself is only an assumption. In any case, however, the canon has been shown to represent some muscle data (Colle and Delville, 1932) and not to represent some nerve data (Lapicque, 1926<sup>2</sup>, Rushton, 1932, Colle and Delville, 1932). It appears, therefore, to be an adequate representation only of special cases of both excitabilities and no definite conclusion can be drawn from its application.

Another method, the one to be used chiefly here, is based on a general property of the excitatory state. There is agreement now, that the excitatory process is not cumulative only, but that it is also dissipative. The rate of the dissipative or subsidence process is measured in some way or other by the various time factors of excitation, the usual measures of excitability. On the other hand, this subsidence is the factor which determines latent addition. Thus, there must be a correspondence between the time factor of excitation and the interval for latent addition which is determined by the rate of subsidence of the excitatory state left by an inadequate stimulus. Therefore, a tissue of long chronaxie must have long latent addition intervals, and *vice versa*. And if for any reason, a tissue of short chronaxie yields a long *pseudo* chronaxie on account of the methods employed, the fault may be discovered by finding a short latent addition interval.

The purpose of the present paper is to discuss briefly the form of the  $\alpha$  strength-duration curve and to show that the  $\alpha$  latent addition times correspond in the same way to the low  $\alpha$  excitability as do the latent addition times in the sciatic nerve to its high excitability.

### *The Strength-Duration Curve*

It has been shown already (Blair 1932 *a*, *b*, 1935 *a*) that some strength-duration data of the  $\alpha$  excitability by Lapicque (1931) and Benoit (1934) conform quite well to the equation,

$$\log \frac{V}{V - R} = kt + \log \frac{K + k\alpha}{K} \quad (1)$$

in which  $V$  is the strength of the stimulus,  $R$  the rheobase,  $t$  the duration of the stimulus, and  $K$ ,  $k$ , and  $\alpha$  are constants. The excitability

<sup>2</sup> Lapicque (1926), pp. 91, 215



of the tissue is measured in the usual way by  $k$ . These curves did not extend to very short durations, however, and with those that do it is necessary to use an additional term as required with nerve (Blair, 1937), i.e. it is necessary to use the equation,

$$\frac{V-R}{V} \times \frac{K+k\alpha}{K} = e^{-kt} + \frac{k\alpha e^{-at}}{K} \quad (2)$$

$a$ , being a constant. This equation is the same as 1 except for the transient term in  $e^{-at}$  which is negligible when  $at$  attains a value of 4 or more as it usually does when the stimuli are longer than about 5 msec. A large number of data have been obtained, which can be used to test the validity of these equations.

#### *Apparatus and Method*

The apparatus for obtaining rectangular waves of direct current has been described previously (Blair, 1935b). In the present instance, the durations of the stimuli apart from the rheobase range from 112 to 4.5 msec. The threshold is obtained using the succession of stimuli whose frequency is given by the speed of rotation of the apparatus, which in this case is about 30 per minute. The 2 second interval is sufficiently long to avoid latent addition and the threshold is not influenced by repeated stimulation as is the case with some slow tissues (Lapicque, 1926, Bonnardel and Goudchaux, 1935).

The tissue, the sartorius muscle of *Rana pipiens*, is dissected the day before the experiment and is kept in Ringer's solution in the cold until about 2 hours before the measurements are made. It is then set up in Ringer's solution in one of two types of glass vessels one of which is illustrated in Fig. 1 and allowed to stand in a water bath at the experimental temperature about 22°C. With this procedure it is found that the preparations usually are quite stable for many hours, as far as the indications of the strength-duration curve are concerned.

Zinc electrodes are used in zinc sulfate solution separated from the experimental vessel by 15 cm. of agar made up in Ringer's fluid. A rather high potential about 200 volts is used in the main circuit to insure low resistance contacts. The current to the tissue is derived from a potentiometer arrangement. The minimal response of the muscle at a point distant from the place of stimulation is used as an index. It is difficult sometimes to get a response from the rheobase which is the same as for the stimuli of limited durations but on the whole it is usually easy to satisfy the requirement that the same part of the muscle is excited by each stimulus throughout the curve. In determining the curves the tissue is considered to be stable if the rheobase and one other point have the same values within about 5 per cent initially and finally.

The vessel illustrated in Fig. 1 is of glass throughout. The rubber stoppers holding the electrodes are kept separated from the solution as indicated. The

tube in which the distal end of the muscle is suspended is closed at the bottom so that the current flows only through the pelvic end, which almost fills the constricted part of the tube. With this arrangement the nerve is never excited.

Usually the right-hand electrode (Fig. 1) is connected to the anode of the battery. This is the current direction used by Rushton (1932*b*) who showed that excitation took place at the cathode in his arrangement. This point has not been studied but it is very probable that this also occurs here and that, therefore, the excitatory wave along the muscle is propagated through the anode when the stimulus is continuous. With the current in the other direction, cathode down, the strength-duration curve is not so easily determined.

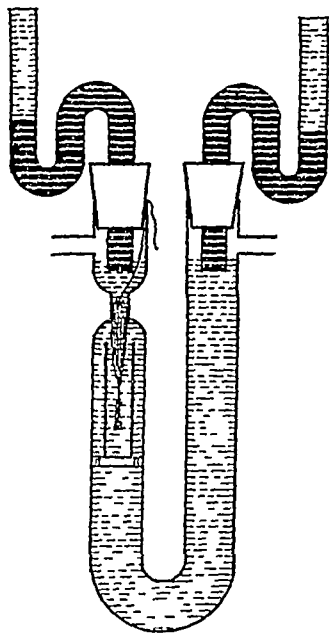


FIG. 1. Apparatus used for  $\alpha$  excitation. The muscle is suspended in Ringer's solution as shown. The electrodes at the top are  $\text{Zn-ZnSO}_4$ -agar in Ringer's solution.

The other type of vessel, which is the one most often used, differs only in that there is no inner tube surrounding the muscle. In this type, part of the current continues through the whole length of the muscle but the current density is so much higher at the constricted portion of the tube than it is lower down that the nerve seldom is excited. With the addition of certain electrolytes, however, in studying salt effects, the excitability of the nerve may become sufficiently high, relative to that of the muscle, that the Lucas type of curve is obtained.

#### EXPERIMENTAL RESULTS

Almost invariably the rheobase or a stronger stimulus, if maintained, evokes a repetitive response. Accommodation of the sort occurring

in nerve is absent therefore, and the recovery of excitability takes place while the current is flowing. As was remarked previously the minimal response to the rheobase is sometimes different from that obtained with other stimuli. Sometimes it is even at a different part of the muscle so that a greater than minimal stimulus must be used to elicit a response at the region which responds to minimal stimuli of shorter durations. Thus the rheobase, as has been remarked by others, *e.g.* Rushton (1930), Lapicque (1931*a*), and Benoit (1934), is not always a very satisfactory measurement. However, with the current directed downward (Fig 1) it is quite stable and easily repeatable so that it usually appears in this case to be quite satisfactory. With the current reversed (cathode down, Fig 1) all the measurements are inclined to be variable. Thus the current directions apparently most suitable are opposite in nerve and muscle.

In Fig 2 are given typical strength duration curves plotted with  $\log \frac{V}{V-R}$  as ordinates and the durations as abscissae, according to equation 1. It is found that the data are of two general types. The first type is illustrated in the lowest curve and the highest, which are linear throughout and which conform closely, therefore, to equation 1. The difference between them is only in the sign of the intercept on the ordinate which is negative in the highest. This type comprises only about 30 per cent of our data and it is not so common as we had been led to expect from data by Lapicque and Benoit (Blair, 1932 *a, b*, 1935 *a*). No numerical comparisons of the data of this type with the equation are given because a number of examples have been considered in the papers just mentioned.

The second type differs from the first in that the rheobase appears too small, as is evinced by a tendency of the points to fall below the line for the long durations. A pair of such curves is given numerically in Table II. It will be seen here that the voltages calculated according to equation 1 are systematically lower than the measured values for durations greater than about 40 msec except for the rheobase which agrees because its measured value is taken as correct. In this type of curve in general the data can be fitted quite well to equation 1 if the rheobase is arbitrarily raised by about 5 per cent taking the rest of the curve as it is measured out to 0.1 second at least. The discrepancy can be laid, therefore, to the rheobase for the convenience of discussion.

and from this point of view some factor enters at long durations which lowers somewhat the threshold current. One suggestion which can be made in this regard is that prolonged currents in nerve do alter the parameters of the strength-duration curve (Nivet, 1934, Blair, 1936 *c*) and also the threshold amount of the local excitatory state, so the longer stimuli in this case may be bringing about these changes in

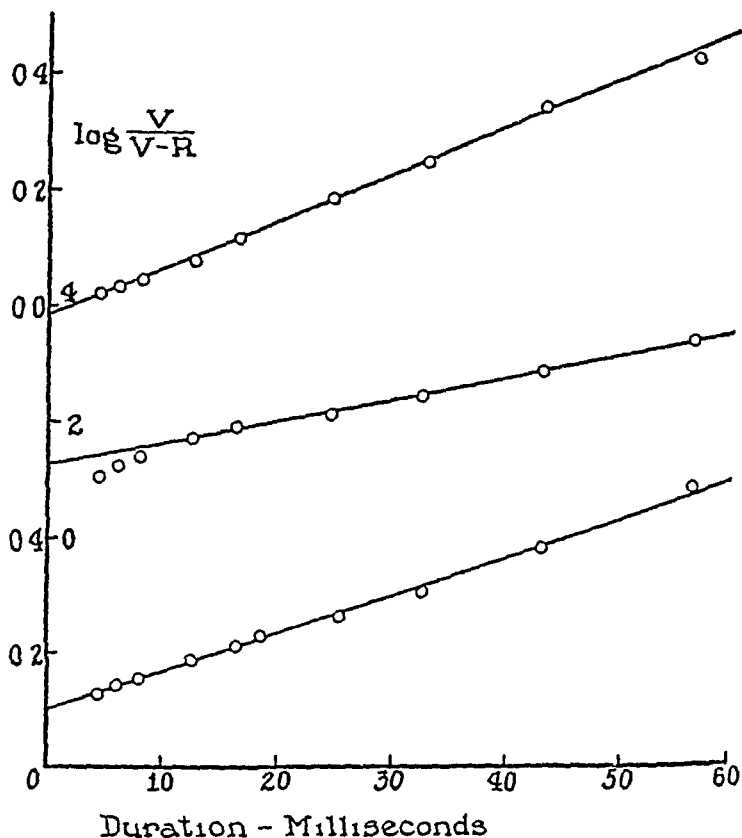


FIG 2 Typical strength-duration curves of the  $\alpha$  excitability plotted according to equation 1. Each curve has a separate ordinate scale.

sufficient amount to account for the discrepancy. Wire electrodes on slow muscle do not, however, show this effect (Blair, 1936 *a*), suggesting that the change of the parameters is associated with the diffuse current distribution of the fluid electrode system and not with the length of the time scale.

At the short-times end of the strength-duration curve, the same

effect is encountered as with nerve (Blair, 1937), *i.e.*, equation 1 becomes inadequate and 2 must be used. This type is illustrated by the middle curve of Fig. 2. In this case, the points diverge downward from the line at about 10 msec but usually the divergence begins at about 5 msec. Therefore, if equation 2 represents these curves it appears that  $e^{-t}$  approaches 0 at about 5 msec. Taking  $at = 5$  at this stage,  $a = 1000$ , which gives the order of magnitude of this constant in sartorius muscle. Since  $k = 20$ , approximately, in this tissue,  $a = 50k$ , approximately.

TABLE I

*Colle's\* Strength Duration Curve from the Frog's Heart*

The calculated voltages are derived from equation (2) and the given constants

Time	$V_{obs}$	$V_{calc}$	
<i>msec</i>	<i>rheobases</i>		
230	1	1	$T = 5 C$
95	1	1.03	$\alpha = 4800$
45	1.09	1.06	$k = 59.1$
21	1.38	1.38	$\frac{K + k\alpha}{K} = 1.04$
10.5	2.06	2.06	
4.1	3.97	4.08	
1.98	6.17	6.93	
0.92	10.6	10.7	
0.41	16.9	17.9	
0.195	28.6	28.6	

\* Experiment C, Colle (1933), p. 419

Since our data do not extend to durations shorter than 4 msec they are not well suited to testing equation 2. Data by Colle (1933) on the frog's heart appear to be very similar to  $\alpha$  data, however. These extend to durations short enough that a large part of the curve diverges from equation 1. One example of these data was given previously (Blair, 1936 c), another set is given here in Table I. This as well as the previous example conforms fairly closely to equation 2. For the heart muscle, according to these data,  $a$  is of the order of 7000,  $k$  of 100, both factors being larger than in the sartorius muscle. Colle's data are not frequent enough for the middle durations to determine well the linear relation of Fig. 1. For this reason, only a few of his sets can be dealt with easily. With his data also, as with the  $\alpha$  excit-

ability, the rheobase tends, sometimes, to be too low for equation 1 or 2. The nature of this transient effect, the term in  $e^{-at}$  of equation 2, which is perhaps purely physical, has been discussed for nerve previously (Blair, 1937) so it will not be considered further here.

It seems possible to conclude from these data and those considered previously that equations 1 and 2 fit the data quite closely in only about one-third of the  $\alpha$  curves, the remainder requiring some additional factor to account for a progressive lowering of the rheobase by about 5 per cent or so. Therefore, it is desirable to examine the indications of the data of latent addition before attempting to reach conclusions as to whether the  $\alpha$  excitation is of the usual form or not.

### *Latent Addition*

As was pointed out above, the fit of equation 1 or 2 to the data, while it provides a strong indication of the validity of the differential equation from which it is derived, is not so direct a test as a method in which the components of the excitatory process can be studied separately. Such a method is provided by a study of the latent addition, for according to the basis of equation 1 or 2 (Blair, 1932, 1936c), if the current is stopped before excitation has been accomplished, the excitatory state,  $p$ , will subside according to

$$\frac{dp}{dt} = -kp \quad (3)$$

$p$  will subside, at a rate proportional to its instantaneous magnitude if the factors giving rise to the term in  $\alpha$  of equation 1 are neglected. During this phase, therefore, the subsidence factor alone is acting and it can be examined separately. This can be done by giving an inadequate stimulus followed after an interval by a test stimulus sufficient to bring about excitation. In the present case, for simplicity, two rectangular stimuli of equal durations are used, the only variables being the interval between them and their strengths which are, however, the same for both.

Letting the stimuli have strengths,  $V$ , and durations,  $t_1$ , it is easily seen on integrating in the usual way (Blair, 1932b) that at the end of the first stimulus,

$$p = \frac{KV}{k} (1 - e^{-kt_1}) \quad (4)$$

$p$  then subsides according to equation 3 from this value to attain, after an interval,  $t_2$ , measured from the end of the first stimulus, a value,

$$p = \frac{KV}{k} e^{-k_2 t_2} (1 - e^{-k_1 t_2}) \quad (5)$$

$k_2$  being used to allow for the  $k$ 's of equations 1 and 3 being different. The second stimulus, to be adequate, must now, during a time,  $t_1$ , raise this value of  $p$  to  $k - \alpha V$  (using the negative sign only) (Blair, 1932 *b*) so that again using equation 1 and substituting the rheobase,  $R$ , for  $\frac{kV}{K + k\alpha}$  there is obtained finally,

$$\frac{V - R}{V} \times \frac{K + k\alpha}{K} = e^{-k_1 t_1} - e^{-k_2 t_1} (e^{-k_1 t_1} - e^{-k_2 t_1}) \quad (6)$$

It will be observed that this applies only to the special case, as was pointed out above, in which the kinetics of the current transient on removing the stimulus are ignored. Actually, if this transient is contra excitatory the process following the inadequate stimulus should be written

$$\frac{dp}{dt} = -\alpha V(a - k)e^{-k_1 t} - kp \quad (7)$$

and after performing the appropriate integrations as before there is finally obtained

$$\frac{V - R}{V} \times \frac{K + k\alpha}{K} = e^{-k_1 t_1} - e^{-(k_1 + k)t_1} - e^{-k_2 t_1} (e^{-k_1 t_1} - e^{-k_2 t_1}) \quad (8)$$

which, however, is the same as 6 when  $t_1$  is greater than about 4 msec since the second member on the right disappears at this stage. It is implied therefore, in 6 that the transient is considered both for excitation and subsidence and that  $t_1$  is sufficiently large to make  $e^{-at}$  negligible. The experiments were done in such a way as to satisfy this requirement.

Equation 6 by itself contains three arbitrary constants,  $\frac{K + k\alpha}{K}$ ,  $k$ , and  $k_2$ . Moreover, it is not easily applied because of its form. However, if a strength duration curve is obtained at the same time, the

first two constants can be derived separately from equation 1. Then, since  $V$ ,  $R$ ,  $t_1$ , and  $t_2$  are measured,  $k_2$  is the only arbitrary constant remaining. Its determination is considered in the next section.

### *Apparatus and Method*

The data of latent addition are obtained by means of the following arrangement. On the same shaft, in addition to the disc used for direct current measurements are mounted two fiber discs 14 cm. in diameter and 1.3 in. in thickness. In the edge of each, through the whole thickness, is a brass segment 2 mm. wide. These segments are connected to each other and by means of a brush, to the same voltage supply and potentiometer as that used for the strength-duration measurements. The other contact from each segment is made by means of a steel bearing ball, 1 cm. in diameter, which is free to turn on lubricated carbon in a brass socket, a spring presses the ball against the edge of the disc. The discs are movable on the shaft so that they may be arranged with the brass segments separated by any desired distance along the circumference. The separation between the segments determines the interval between the stimuli, and the durations of the stimuli are fixed by the widths of the segments, since the balls make point contacts. The threshold for the two stimuli is determined by trial adjustment of the potentiometer while the discs are rotating continuously. As the time of rotation is 2 seconds, there should be no latent addition between successive pairs of stimuli and in fact a double response or none at all is obtained with intervals of 200 msec. or even less. The latent addition determinations are preceded and followed by the obtaining of a strength-duration curve. These curves provide an independent index of the stability of the tissue as well as the constants  $\frac{K + k\alpha}{K}$  and  $k$ .

Equation 6 is applied by calculating the constant  $e^{-k_1 t_1}$  using  $k$  from the strength-duration curve and the measured duration of the stimulus,  $t_1$ . The factor,  $e^{-k_2 t_2}$  can have values from 0 to 1 only, the respective limits being when the latent addition interval,  $t_2$ , is very long and zero. A graph of  $\frac{V - R}{V}$  as ordinates and  $e^{-k_1 t_1}$  as abscissae must therefore have an ordinate intercept  $e^{-k_1 t_1} \cdot \frac{K + k\alpha}{K}$  when  $t_2$  is large, i. e. when  $e^{-k_2 t_2} = 0$  and an ordinate  $e^{-k_1 t_1} \cdot \frac{K + k\alpha}{K}$  when  $t_2 = 0$ , i. e., when  $e^{-k_2 t_2} = 1$ . These ordinates are marked, therefore, on a graph at the point  $e^{-k_1 t_1} \cdot \frac{K + k\alpha}{K} = 0$  and 1, respectively, and are joined by a straight line. Now each experimental value of  $\frac{V - R}{V}$  will give by means of the graph, a corresponding value of  $e^{-k_2 t_2}$ . From this  $k_2 t_2$  can be calculated and then  $k_2$ , because  $t_2$  is measured. This procedure is followed for several data and the resulting values are used to obtain an average value for  $k_2$ , if there is one of the kind required by the hypothesis. The fit of equation 6 can then be tested by calculating  $V$  for each interval and



comparing it with the measured value. Or the calculated values of  $e^{-kt_1}$ , using the average  $k_2$  can be plotted on the graph used previously as a graphic indication of the fit

TABLE II

*Latent Addition Data (Lower Set) on the  $\alpha$  Excitability Preceded and Followed by a Strength-Duration Curve (Upper Set)*

Duration msec	Initial		Final		
	$V_1$	$V_{calc}$	$V_2$	$V_{calc}$	
$\infty$	450	450	450	450	Initial curve
112	530	505	550	505	$k = 17.75$
74.2	600	580	600	580	$\frac{K + k\alpha}{K} = 1.217$
56.3	670	645	670	650	
43	750	730	760	735	
32.7	850	835	860	845	Final curve
25.4	950	940	960	965	$k = 17.95$
18.6	1080	1095	1100	1130	$\frac{K + k\alpha}{K} = 1.19$
16.4	1150	1165	1160	1200	
12.5	1370	1315	1380	1365	
8.0	1650	1565	1680	1655	Mean $k = 17.85$
6.1	1740	1715	1860	1820	$\frac{K + k\alpha}{K} = 1.205$
4.5	1850	1915	1950	2000	
$\infty$	450		450		
Interval		$V_{obs}$	$V_{calc}$		
msec					
213		1540	1545		
4.26		1150	1185	Durations, $t_1 = 8.8$ msec	
8.52		1210	1220	$e^{-kt_1} = 0.855$	
12.78		1260	1265	$e^{-2kt_1} = 0.730$	
17.05		1300	1300	$k_2 = 38.1$	
21.3		1350	1330		
34.05		1430	1400		
42.6		1490	1440		
51.2		1480	1450		
63.9		1540	1495		
106.5		1550	1530		
127.8		1560	1545		
213		1550	1545		

## EXPERIMENTAL RESULTS

In Table II are given the data of a typical experiment which include an initial strength-duration curve, a set of latent addition measure-

ments, and a final strength-duration curve. The constants from each strength-duration curve are given. These are used separately to obtain the calculated voltages of the strength-duration curves, and their mean values are used in equation 6 for the latent addition calculations. In Fig 3 (curve B) are plotted  $\frac{V-R}{V}$  against  $e^{-k_2 t_2}$  for these data. These values of  $e^{-k_2 t_2}$  are calculated from a mean value of

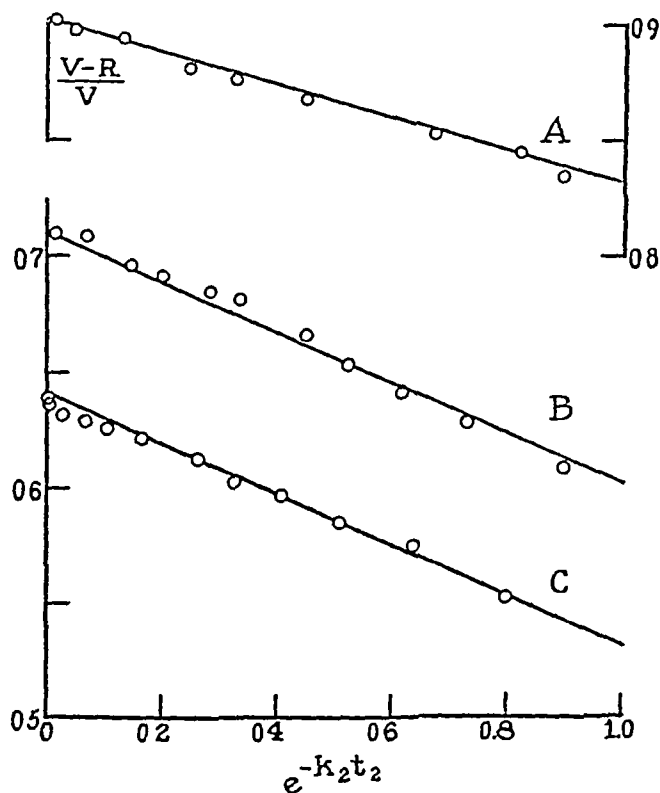


FIG 3 A method of plotting latent addition data according to equation 6 which predicts a linear relation. Curve B is for the data of Table II. Curves A and C are for sets A and C, respectively, of Table III.

$k_2$  obtained previously from the same curve by the method given above. It will be seen that these data conform to equation 6 in fair approximation. In Table III are given three other examples of the latent addition data alone as measured and as calculated, using again the constants from strength-duration curves obtained at the same time. In Fig 4 are given the curves of subsidence of the excitatory

state for the data of Table II, curve A, and for sets B and C of Table III, curves B and C. These curves as drawn are the theoretical curves of subsidence using the mean value of  $k_2$ . The plotted points are the experimental values calculated from the measured voltages

TABLE III

*Typical Data for the Latent Addition of  $\alpha$  Excitation*

The intervals are the intervals between the stimuli,  $V$ , whose durations are  $t_1$ . The calculated voltages are from equation (6) using the constants given

Interval <i>msec</i>	A		B		C	
	$V_{obs}$	$V_{calc.}$	$V_{obs}$	$V_{calc.}$	$V_{obs}$	$V_{calc.}$
213			240	237	1880	1895
4 26	2100	2110	207	203	1520	1525
8 52	2220	2255	213	212	1600	1590
12 78			219	218	1640	1645
17 05	2470	2540	225	224	1690	1685
21 3	2600	2640	229	227	1710	1725
25 51			230	230	1750	1750
29 8	2760	2820				
34 05			231	233	1790	1805
42 6	3020	3010	234	236	1810	1840
51 2			236	237	1830	1860
63 9	3190	3120				
68 2					1850	1880
85	3240	3180	237	237		
106 5					1870	1895
128	3260	3280	237	237		
213	3270	3280	237	237	1890	1895
	$t_1 = 4.6 \text{ msec.}$		$t_1 = 8.8 \text{ msec}$		$t_1 = 8.8 \text{ msec}$	
	$R = 325$		$R = 120$		$R = 680$	
	$k_1 = 16.0$		$k_1 = 31.4$		$k_1 = 21.4$	
	$k_2 = 47.5$		$k_2 = 81$		$k_2 = 53$	
	$\frac{k_2}{k_1} = 2.94$		$\frac{k_2}{k_1} = 2.57$		$\frac{k_2}{k_1} = 2.46$	
	$\frac{K + k\alpha}{K} = 1.026$		$\frac{K + k\alpha}{K} = 1.533$		$\frac{K + k\alpha}{K} = 1.29$	

by means of equation 6, the excitatory state being assumed in each case to have the value unity at the end of the first stimulus

It will be seen on considering the graphs and the tables together that in no case is the percentage divergence of the experimental from the

theoretical value greater than 3 and usually less than 2. All of our data are similar. It is concluded, therefore, from these results, that the subsidence of the excitatory state is exponential in close approximation.

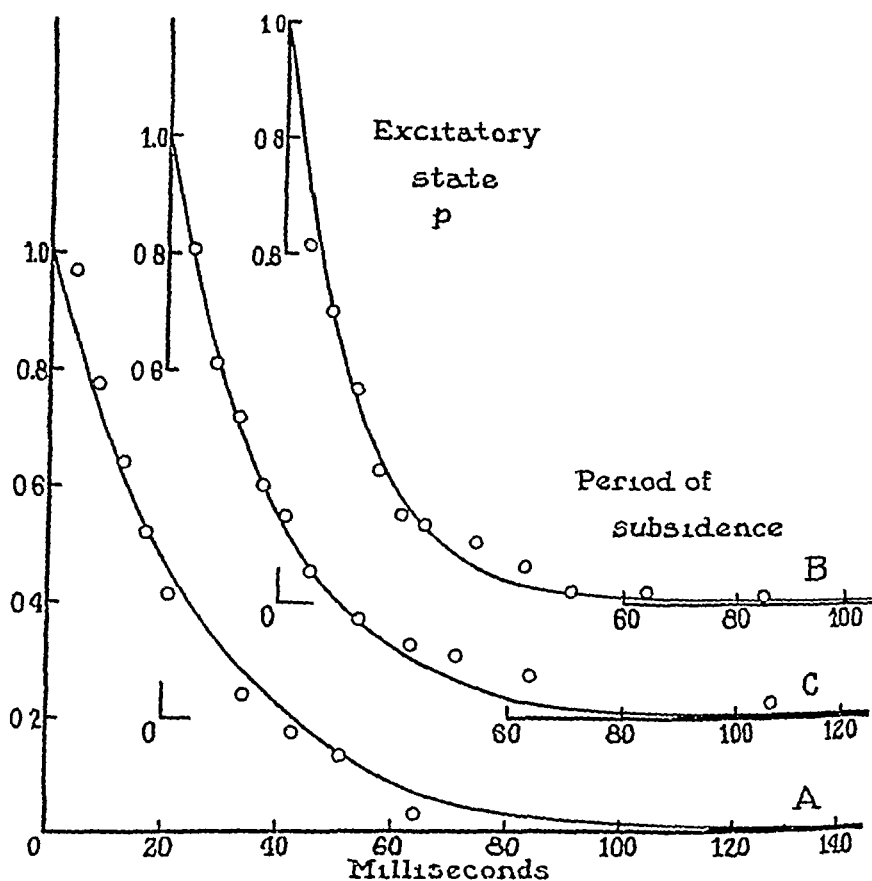


FIG. 4. The subsidence of the  $\alpha$  excitatory state. It is assumed for convenience that the first (inadequate) stimulus produces unit value of the excitatory state so the maximum ordinate is 1. Curve A represents the data of Table II. Curves B and C represent sets B and C, respectively, of Table III.

It will be observed, however, that  $k_2$  is more than twice as great as  $k_1$ . That is, the subsidence is more than twice as fast when examined directly as it appears to be from the strength-duration curve. This result was quite unexpected and it was thought at first to be a feature of the  $\alpha$  excitability only. Preliminary work on nerve gives, however, a similar result (Blair, 1936c). There appear to be two alternative explana-

tions of this result, either that the mechanism postulated to give equations 1 and 2 is quite wrong or that the excitatory state is dissipated by factors in addition to those involved directly in the process of excitation. It appears to be most probable at present that the latter alternative is the correct one (Blair, 1936 *c*) but the matter is open to question.

That the particular form of stimulus is not a factor in the determination of this ratio for single pulses in nerve, at least, is indicated by the finding that the same value of  $k$  is derived using either rectangular pulses, condenser discharges, or linearly rising currents (Blair, 1935 *b, c*) as stimuli.

### *The Refractory Period*

The recent theoretical treatments of excitation (Rashevsky, 1933, Monnier, 1934) relate the refractory period to the time constants of excitation and accommodation. This point of view has received no experimental justifications but there is perhaps some parallelism, usually, between the length of the chronaxie and the refractory period. This is not true of the  $\alpha$  excitability, however. In this case preliminary measurements give the absolute phase as being of the order of 1 msec at 20°C which is somewhat shorter than most of the sciatic nerve data. This is in agreement with the assumption which has always been made by those using the muscle as an index while measuring the refractory period of nerve, namely, that the refractory period of the muscle must be as short or shorter to permit the method. As the matter stands at present there does not seem to be sufficient evidence of a connection between the strength duration curve and the refractory period to draw a conclusion with respect to the question at issue.

### CONCLUSIONS

Apart from this new problem which is raised by the dissimilarities of the subsidence rates during and following the stimuli, it is apparent that the  $\alpha$  latent addition data are quite as appropriate as regards their time scales to the  $\alpha$  strength duration curves with their small  $k$  values, or large chronaxies as the latent addition data in nerve are to the large  $k$  values or short chronaxies encountered there. Thus it

can be concluded that the  $\alpha$  process of excitation is essentially the same in its general nature as those occurring in other tissues and that there is no reason to believe that any information derived from its study will be any more or less distorted than that obtained elsewhere. It may be, in fact, that the  $\alpha$  excitation process is the simplest because it does not involve any of the accommodation which occurs so frequently in other cases and which probably distorts somewhat the strength-duration curve.

#### SUMMARY

With a view to indicating that the  $\alpha$  excitatory state in muscle is not of a special nature it is shown that the  $\alpha$  strength-duration curves are of the same form as those determined for nerve and other tissues except that in about two-thirds of all cases the rheobase appears to be slightly too low. Also from experiments in latent addition it is found that the  $\alpha$  excitatory state following an inadequate stimulus subsides exponentially at a rate which is related to the  $\alpha$  excitability in the same way, approximately, as the subsidence rate in nerve is related to the nerve's excitability. In both tissues the subsidence as measured directly is 2-3 times as fast as it appears to be from the strength-duration curve. The  $\alpha$  refractory period is at least as short as that of nerve so the  $\alpha$  chronaxie is unusually long compared to the refractory period. There is no reason at present, however, to consider this as having any bearing on the problem at issue. It is concluded therefore, that the  $\alpha$  excitability differs from others in the rates of its reactions rather than in its fundamental nature and that any conclusions about excitability drawn from its study will probably be valid quite generally.

I am indebted to Mr W B Latchford for assistance in doing the experiments.

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## TRANSFORMATION OF SWINE PEPSINOGEN INTO SWINE PEPSIN BY CHICKEN PEPSIN

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Previous work (1) has shown that the transformation of swine pepsinogen to swine pepsin at pH 4.0-5.0 is an autocatalytic reaction, *i.e.*, the pepsin forms itself from pepsinogen. It has also been found (2) that swine pepsin and chicken pepsin are distinct immunologically and that they differ in addition by the fact that chicken pepsin is much less sensitive to alkali than swine pepsin. The question arises as to whether swine pepsinogen activated by chicken pepsin would result in the formation of chicken pepsin or swine pepsin. It would be expected that the species specificity of the enzyme was already present in the inactive precursor and that the formation of the active group in this inactive molecule would be without effect on the species specificity. If this were the case swine pepsinogen would be transformed to swine pepsin no matter whether the reaction were catalyzed by swine pepsin or chicken pepsin and, conversely, chicken pepsinogen would be transformed to chicken pepsin whether the reaction were catalyzed by chicken pepsin or swine pepsin.

In order to determine the result of such an experiment swine pepsinogen has been activated by the addition of chicken pepsin and it has been found that swine pepsin is formed under these conditions. Similarly, chicken pepsinogen when transformed into the active enzyme by swine pepsin gives rise to chicken pepsin. In a sense, therefore, it may be said that the swine pepsin becomes "adapted" when added to chicken pepsinogen since instead of forming more swine pepsin, as would be the case were it added to swine pepsinogen, it now forms chicken pepsin. It has been suggested (3) that the formation of bacteriophage and possibly the viruses is analogous to the for

mation of active enzymes from their precursors and the results of the present experiments are formally, at least, analogous to the adaptation of bacteriophage to a different host organism

### EXPERIMENTAL RESULTS

If an alkaline solution of pepsinogen is brought to pH 4.6 it is slowly transformed into active pepsin and the course of the reaction in general follows that of an autocatalytic reaction. The reaction is presumably initiated by traces of pepsin already present since it has not been possible to prepare pepsinogen completely free of pepsin activity. In order to determine the effect of the addition of pepsin upon the rate of activation, therefore, it was necessary to compare the rate of activation of the pepsinogen solution alone with that of a solution to which had been added active pepsin. Owing to the peculiar nature of the autocatalytic reaction it is necessary to add large amounts of active pepsin in order to markedly affect the activation curve. The autocatalytic equation predicts that the most striking difference will be found by comparing the rate of activation of the pepsinogen alone with that of a solution to which has been added about an equivalent amount of active pepsin. The experiments were therefore carried out by comparing the rate of activation of a solution of pepsinogen which, after activation, would have an activity of about 15 rennet units per ml, with that of a similar solution to which had been added sufficient swine or chicken pepsin to bring the initial activity to 15 rennet units per ml. The total increase in activity of the two solutions is therefore the same. The experiments were carried out in  $M/1$  pH 4.6 acetate buffer at  $25^{\circ}\text{C}$ . Samples were taken into  $M/1$  pH 5.6 acetate buffer at various time intervals and also in  $0.4 M$  pH 8.5 borate buffer. In the latter solution swine pepsin is completely inactivated in about 5 minutes, whereas chicken pepsin is not inactivated appreciably for at least  $\frac{1}{2}$  hour. 0.5 cc of the pH 5.6 acetate samples and also of the pH 8.5 borate samples (after titration to pH 5.0) were added to 5 cc of a standard "Klm" (4) solution and the time of clotting determined at  $37^{\circ}\text{C}$ . The activity, as determined from the acetate sample is called "total rennet units" and that from the borate sample is called "chicken rennet unit". One rennet unit is defined as the quantity of enzyme which will clot 10 cc of a standard 20 per cent Klm solution in 1 minute at  $37^{\circ}\text{C}$ .

An outline of the method of preparation of chicken pepsinogen is given in Table I and of chicken pepsin in Table II.

The result of the experiment in which swine pepsinogen was activated by chicken or swine pepsin is shown in Fig. 1 in which the increase of total pepsin and of chicken pepsin is plotted against the time of activation. The results show that the pepsinogen solution to which chicken or swine pepsin has been added activates much

more rapidly than the pepsinogen solution alone and also that the increase in activity is entirely swine pepsin and that no new chicken pepsin is formed

In Fig 2 the results have been plotted as the log of  $\frac{A - A_0}{A}$  against the time where  $A_0$  is the final activity and  $A$  is the activity at time  $T$ . This method of plotting gives a straight line for the two experiments

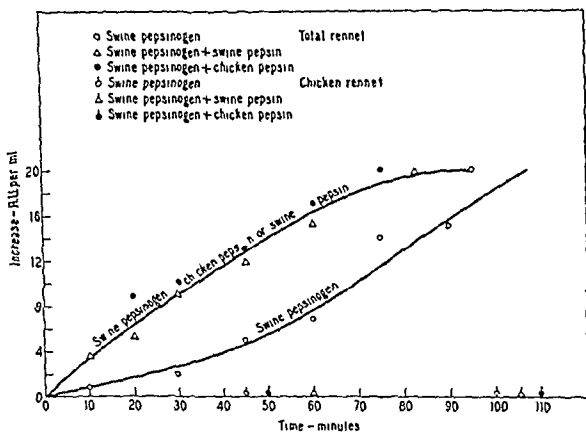


FIG 1 Effect of the addition of chicken pepsin on the formation of pepsin from swine pepsinogen

showing that the reactions are autocatalytic and have about the same value for the autocatalytic constant. This shows that chicken pepsin is catalytically as effective as swine pepsin in the activation of swine pepsinogen. A similar experiment in which chicken pepsinogen was activated with swine or chicken pepsin is shown in Fig 3. The results show again that chicken pepsin is formed from chicken pepsinogen whether the transformation is brought about by swine pepsin or chicken pepsin since in this case the increase in activity, from measurements of the samples which had stood at pH 8.5, is the same as that obtained from the pH 5.6 acetate sample.

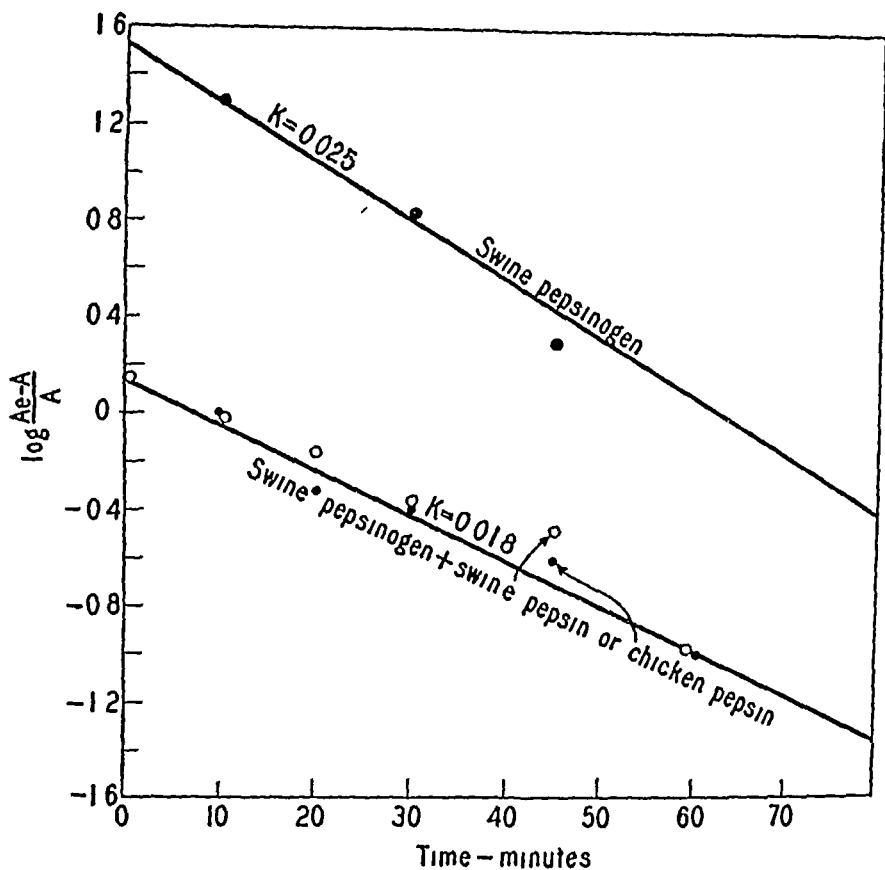


FIG 2 Activation of pepsinogen shown in Fig 1 plotted in accordance with the autocatalytic equation, i.e.,  $\log \frac{A_e - A}{A}$  against the time

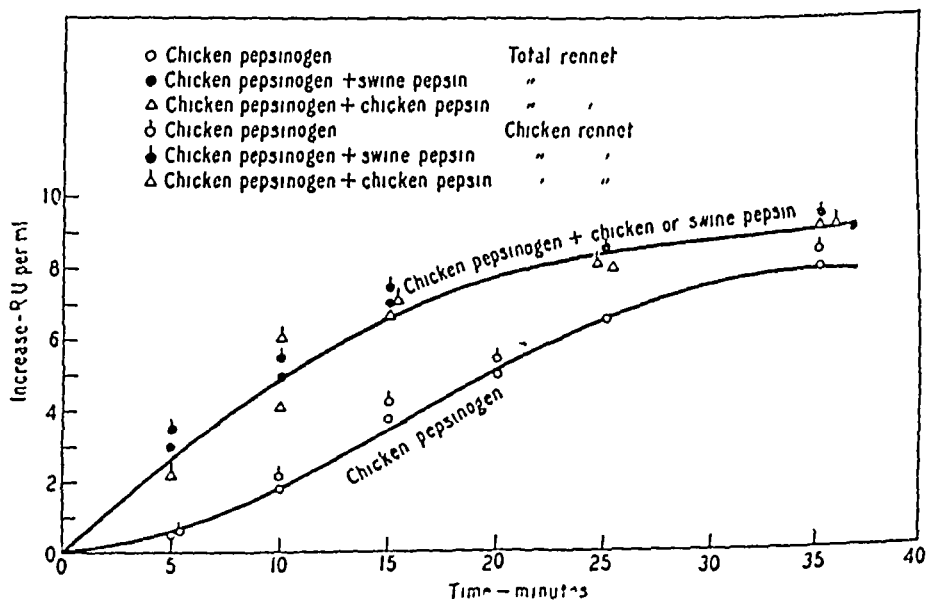


FIG 3 Effect of chicken or swine pepsin on the transformation of chicken pepsinogen to chicken pepsin

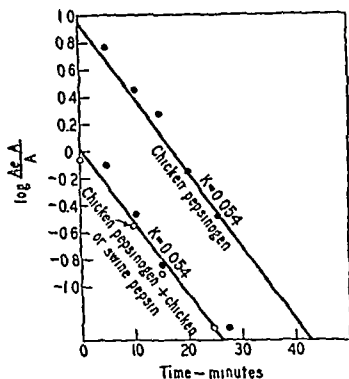


FIG 4 Activation of pepsinogen shown in Fig 3 plotted in accordance with the autocatalytic equation:  $\log \frac{A - A_0}{A}$  against the time

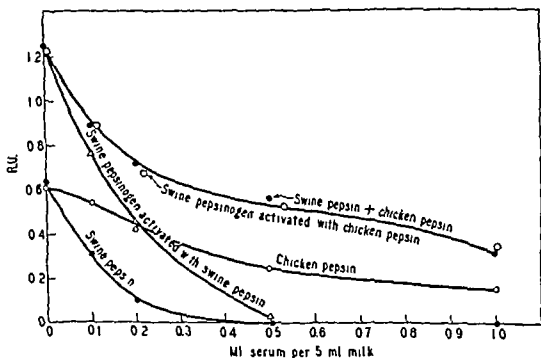


FIG 5 Effect of serum on the rennet activity of various mixtures of swine and chicken pepsin

In Fig 4 the results have been plotted against the log of  $\frac{A_0 - A}{A}$  and again show fair agreement with the course of an autocatalytic reaction In this experiment the final concentration of pepsin was

TABLE I  
*Outline of Method for Partial Purification of Chicken Pepsinogen*

Procedure	No	Carbohydrate (as glu cost)	[P U]Hb*	
			total	1/m <sub>s</sub> N
423 stomachs collected in 0.45 sat ammonium sulfate — $\pi/10$ sodium bicarbonate solution Supernatant discarded	1			
Stomachs defatted and minced, stirred 12 hrs with a solution containing 1102 ml $\pi/1$ sodium bicarbonate, 1745 ml sat ammonium sulfate, and 8178 ml water, final concentration of ammonium sulfate is about 0.2 saturated 10 per cent Filter Cel and 5 per cent Hyflo Super Cel added and mixture filtered and washed twice with 0.2 sat ammonium sulfate $\pi/10$ sodium bicarbonate solution Filtrate re-filtered after addition of 1 per cent Filter Cel Filtrate	2	5950	367	0.032
Added 188 gm ammonium sulfate per liter Precipitate filtered with Hyflo Super Cel and dissolved in 8.5 liters 0.01 $\pi$ sodium bicarbonate	3	1672	330	0.051
Added equal volume sat ammonium sulfate dropwise to solution No 3 Precipitate filtered with aid of Hyflo Super Cel Dissolved in 10 volumes 0.01 $\pi$ sodium bicarbonate	4	1080	323	0.065
Solution No 4 made pH 6.0 with 4 $\pi$ pH 4.65 acetate Added 1.25 volumes of pH 6.0 $\pi/1$ copper hydroxide, filtered Precipitate extracted with 3000 ml $\pi/10$ dipotassium phosphate Filtered Copper hydroxide residue washed twice with 420 ml phosphate Filtrate	5	300	262	0.19
1256 gm ammonium sulfate added to solution No 5 Precipitate removed by filtration with suction Cake dissolved in distilled water, total volume being one-half of No 4	6			
Solution No 6 adjusted to pH 6.0 1.25 volumes of pH 6.0 $\pi/1$ copper hydroxide added Filtered Precipitate extracted with 1500 ml dipotassium phosphate, filtered Copper hydroxide washed twice with 180 ml phosphate Filtrate	7	140	172	0.19

\* Activity after transformation into pepsin in acid solution

half that of the first experiment and the autocatalytic constant is correspondingly lower

The conclusion that swine pepsin is formed from swine pepsinogen

may be confirmed by determining the effect of normal rabbit serum upon the activated solutions. It has been previously found that normal rabbit serum inhibits the action of swine pepsin more markedly than it does chicken pepsin. The results of adding increasing amounts

TABLE II  
*Outline of Method for Partial Purification of Chicken Pepsin*

Procedure	No	Carbo- hydrate (as glu- cose)	p U /lb	
			total	I/mg N
398.5 gm crude pepsinogen (No 4 of Table I) dissolved in 3.985 ml 0.01 sodium bicarbonate	1	2.596	616	0.063
Solution No. 1 made pH 6.0 with 4 M pH 4.65 acetate buffer. Added 1.25 volumes pH 6.0 1/1 copper hydroxide. Filtered. Precipitate extracted with 500 ml M/10 dipotassium phosphate. Filtered. Precipitate washed twice with 500 ml M/10 dipotassium phosphate. Filtrate	2	486	400	0.17
31.4 gm ammonium sulfate added to each liter of solution No. 2. Precipitate allowed to settle. Supernatant decanted. 10 gm. Hyflo Super Cel added and precipitate filtered by suction on hardened filter paper. Cake stirred in 2 volumes water. Filtered. Residue washed with 1.5 volumes. Filtrate	3		402	0.17
1 volume 0.1 N hydrochloric acid added to solution No. 3. stood at room temperature for 30 minutes	4		402	0.27
Adjusted to pH 3.4 with 4 M acetate buffer. Cooled to -10 C. Added equal volume cold acetone	5			
Filtered by suction. Discarded precipitate. Filtrate	6		303	0.23
10 volumes water added to solution No. 6. 4 gm Filter Cel added. After stirring suspension filtered on No. 3 Whatman filter paper by suction. Clear filtrate	7		298	
Solution adjusted to pH 3.0 with N/2 sulfuric acid and equal volume saturated magnesium solution added. pH readjusted to 3.0 with 5 N sulfuric acid. Stood in cold room for 2 days. Added 20 gm Filter Cel. Filtered by suction on No. 3 Whatman filter paper. Filtrate discarded	8			
Precipitate extracted with N/10 sodium acetate	9	133	310	0.29

of normal rabbit serum on the time required for swine pepsin or chicken pepsin to clot 5 cc of milk are shown in the two lower curves of Fig. 5. It may be seen that increasing amounts of serum inhibit the clotting due to swine pepsin much more than that due to chicken

pepsin    The two upper curves show the effect of increasing amounts of serum on the clotting of milk caused by a solution of swine pepsinogen activated by swine pepsin and of a solution of swine pepsinogen activated with chicken pepsin. If the activation of swine pepsinogen with an equivalent amount of chicken pepsin results in the formation of swine pepsin the resulting solution should contain equal amounts of swine and chicken pepsin. The results show that the inhibiting effect of serum on swine pepsinogen activated by chicken pepsin is the same as that on a solution made up by mixing equal amounts of swine pepsin and chicken pepsin and is less than the effect on the solution containing only swine pepsin.

#### SUMMARY

Activation of swine pepsinogen with chicken pepsin results in the formation of swine pepsin.

Activation of chicken pepsinogen with swine pepsin results in the formation of chicken pepsin.

The structure responsible for the species specificity of the enzyme is therefore present in the inactive precursor.

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# ELECTRIC IMPEDANCE OF FERTILIZED ARBACIA EGG SUSPENSIONS\*

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(Accepted for publication, November 22, 1937)

In previous work on the alternating current impedance of *Arbacia punctulata* egg suspensions (Cole and Cole, 1936b), it was found that the unfertilized egg had a static membrane capacity of  $0.73 \mu\text{f}/\text{cm}^2$  which was independent of the frequency. On fertilization, the low frequency membrane capacity increased to  $3.1 \mu\text{f}/\text{cm}^2$  and at intermediate frequencies there was evidence of another capacity element. It was suggested that the fertilization data could be interpreted either by an increased plasma membrane capacity which varied with frequency or by an added high capacity of the fertilization membrane. The latter picture was considered the more probable one, and diagrams of the current flow were subsequently published (Cole, 1937). The present work was undertaken to determine whether or not this explanation was correct.

## Measurements

The impedance of the suspensions was measured over the frequency range from 1 kc (kilocycle per second) to 10 mc (megacycles per second) with an alternating current Wheatstone bridge which has been described (Cole and Curtis, 1937).

For the first eggs available in June, the method of preparation of the suspensions and the impedance cell in which they were measured were the same as had been used in the previous work. The membranes did not consistently have  $90^\circ$  phase angles, which would indicate static capacities, but varied from  $87^\circ$  to  $90^\circ$  for the unfertilized eggs and from  $85^\circ$  to  $90^\circ$  for the fertilized eggs. Furthermore, the

\* Aided by a grant from The Rockefeller Foundation.

intermediate frequency effect which had been at its height at 512 kc (see Fig 2, Cole and Cole, 1936 *b*) was much less pronounced

It was then thought that the eggs might have been injured by handling and a new impedance cell was designed to minimize this possibility. This cell, shown in Fig 1, is essentially a glass centrifuge tube with two platinized platinum electrodes near the bottom. Its total volume is 13.3 cc and the volume of the impedance cell proper, with a cell constant of  $12.58 \text{ cm}^{-1}$ , is 0.8 cc. Since the diameter of the electrodes is somewhat larger than the internal diameter of the tube

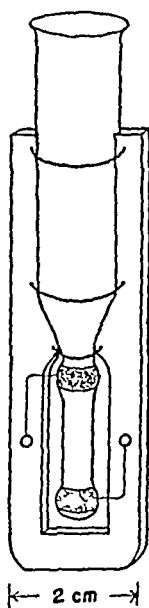


FIG 1 Impedance cell described in text

between them, the electrode polarization correction is considerably diminished at the cost of an increased volume. The cell is wired and cemented into a Victron holder which allows it to fit into a standard centrifuge cup. Washed dilute suspensions of the eggs were transferred to the cell, allowed to settle, and then centrifuged very lightly to minimize the time required for steady low frequency resistance and capacity values to be attained.

Although suspensions were used when inseminated samples showed over 90 per cent fertilization membranes, they were nearly always over 95 per cent. After measurement, the suspensions were diluted and a

similar viability and satisfactory development found. As noted before, in the volume concentrations usually used, 30 to 50 per cent, the fertilized eggs will remain in the single cell stage for a considerable period and yet proceed normally to at least the 16 cell stage when diluted at the end of a run.

After this change in technique, the membrane phase angles of both the unfertilized and fertilized eggs were, with a single exception, found to be  $90^\circ$  at low frequencies as shown in Figs 2 and 3. It will be seen that for the fertilized suspension of Fig 3, although the points corresponding to frequencies above 100 kc still show a definite departure from the semicircle which corresponds to a single capacity element, the divergence is much less than had been found previously (see Fig

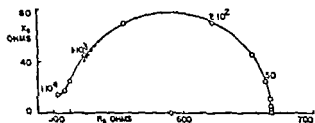


FIG 2

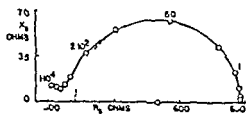


FIG 3

FIG 2 Impedance locus, series resistance,  $R_s$  vs series reactance,  $X_s$ , for a 52.1 per cent volume concentration suspension of unfertilized *Arbacia* eggs in sea water. Frequencies indicated are in kilocycles per second.

FIG 3 Impedance locus, series resistance,  $R_s$  vs series reactance  $X_s$  for a 50.3 per cent volume concentration suspension of fertilized *Arbacia* eggs in sea water. Frequencies indicated are in kilocycles per second.

2, Cole and Cole, 1936 b). Thus it seems that the interpretations which were based on this characteristic in the earlier data are probably not applicable. In a more thorough investigation, the principal problem is the definite localization of the high capacity membranes in the unfertilized and fertilized eggs. Such membranes may be expected to have relatively high electrical resistances and, with this initial assumption, their positions can be determined from volume concentration and low frequency measurements of the suspensions.

#### *Volume Concentration of Suspensions*

When the suspended eggs are enclosed by a poorly conducting membrane, the low frequency resistance of the suspension,  $r_0$ , is the same

as it would be if the eggs were solid non-conductors, and is given by the Maxwell formula,  $\rho_0 = 2 (1 - r_1/r_0)/(2 + r_1/r_0)$ , where  $r_1$  is the resistance of the medium and  $\rho_0$  is the non-conducting volume concentration. For a cubic centimeter of suspension containing  $n_0$  eggs,  $\rho_0 = n_0 v_0$ , where  $v_0$  is the volume enclosed by the non-conducting surface of a single egg. If the volume concentration,  $\rho$ , of this same suspension is determined by another method,  $\rho = n_0 v$  where  $v$  is the volume defined by the method, and then of course,  $\rho/\rho_0 = v/v_0$ . For the dextrose method, used in the work on *Hipponoe* eggs (Cole, 1935),  $\rho/\rho_0$  was very close to unity, so that the volume enclosed by the ion impermeable membrane was practically identical with that enclosed by the dextrose impermeable membrane. Although it is generally believed that the fertilization membrane is freely permeable to both ions and dextrose, and that the plasma membrane is not, it is obvious that the dextrose method alone does not give pertinent information, and that one depending upon microscopic observations on the eggs must be used. The following procedure was used. After the impedance measurements had been made, the suspension was weighed and its volume computed with an assumed egg density of 1.09 gm/cc (Harvey, 1932). The suspension was diluted with 250 cc of sea water and the number of eggs in small fractions counted as suggested by Parpart (Shapiro, 1935) to give  $n_0$ . The average plasma membrane enclosed volume,  $v_p$ , and fertilization membrane enclosed volume,  $v_f$ , were calculated from measurements of the respective diameter in fifty eggs, and expressed in terms of the non-conducting membrane enclosed volume,  $\rho_0$ , by  $v_p/v_0$  and  $v_f/v_0$ . The rather unsatisfactory sampling technique is probably largely responsible for the wide spread of the results which are given in Fig. 4. The plasma membrane enclosed volume averaged 1.6 per cent less than the non-conducting membrane enclosed volume in ten unfertilized suspensions, and 2.6 per cent less in twenty fertilized suspensions, while the fertilization membrane enclosed space averaged 32 per cent greater than the non-conducting membrane enclosed volume.

#### *Membrane Capacity*

The membrane capacities,  $C_M$ , have been determined for eleven suspensions of unfertilized and seventeen suspensions of fertilized eggs by the equation (Cole, 1928 a),

$$C_M = \frac{2C_0k}{\left(2 + \frac{r_1}{r_0}\right)\left(1 - \frac{r_1}{r_0}\right)a}$$

Here  $k$  is the cell constant of the impedance cell,  $r_1$  the resistance of the sea water, and  $r_0$  the low frequency parallel resistance of the suspension,  $a$ , the radius of the plasma membrane.  $C_0$ , the low frequency parallel capacity of the suspension due to the membrane was found by the method used previously (Cole and Cole, 1936 *a*). The distribution of these capacities is shown in Fig 5. We are quite

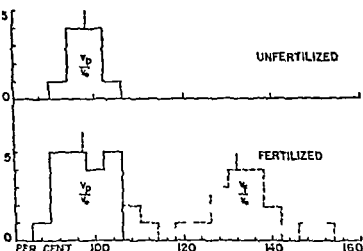


FIG 4

FIG 4 Frequencies per 4 per cent interval of the ratios of the plasma and fertilization membrane enclosed volumes,  $v_p$  and  $v_f$ , to the non conducting membrane enclosed volume  $v_0$  for unfertilized and fertilized suspensions. The mean values are indicated above each group.

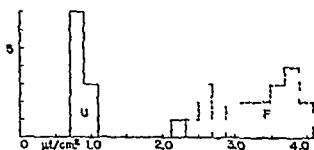


FIG 5

FIG 5 Frequencies per  $0.2 \mu\text{f}/\text{cm}^2$  interval of the membrane capacities of unfertilized,  $U$ , and fertilized,  $F$ , eggs.

unable to explain the high capacity of one unfertilized batch of eggs which was the last of the season. However, ignoring this value, we obtain average membrane capacities of  $0.86 \mu\text{f}/\text{cm}^2$  before fertilization and  $3.3 \mu\text{f}/\text{cm}^2$  after fertilization.

#### Internal Resistance

The internal resistances of the eggs may be estimated as has been done previously (Cole, 1935) from the high frequency extrapolation obtained by neglecting the highest frequency points as indicated by the dotted lines in Figs 2 and 3.

For the unfertilized eggs an average value of 5.7 times the resistance of sea water is obtained from ten suspensions. Twenty fertilized suspensions gave 7.2 times sea water.

### *Interpretation and Discussion*

The non-conducting volumes of both the unfertilized and fertilized eggs were slightly larger than the plasma membrane volumes even though the outermost visible edge of the latter membranes was measured. This fact and a statistical analysis both give us reason to believe that the differences involved are within the experimental limits, and one may then say that the high resistance membrane is either at, or very close to, the plasma membrane in both the unfertilized and fertilized egg. Both because the fertilization membrane volume is significantly larger than the non-conducting volume and because the latter is little, if any, different from the plasma membrane volume, it is quite improbable that the resistance of the fertilization membrane and perivitelline space is appreciably different from sea water. As a consequence, it seems practically impossible for the fertilization membrane to show a measurable capacity. The capacity change on fertilization must then take place at or very near the plasma membrane, and the picture previously presented is untenable. On fertilization it might be assumed either that one of two closely adjacent membranes became completely permeable or that the structure of a single membrane so altered as to increase its capacity. But there seems to be no basis for a choice between these two and probably several other possibilities.

We are still faced with the unpleasant necessity of explaining the large intermediate frequency effect in the earlier fertilized egg data, and, for that matter, the smaller effect which still persists. The simplest and most obvious explanation is that the fertilized suspensions were not homogeneous, but contained unfertilized eggs or eggs having unfertilized membrane capacities. Some support is given to this explanation by the data on a single fertilized *Arbacia* egg (Cole and Curtis, 1938, Fig. 3), which showed no intermediate frequency effect. Calculation shows that in the earlier work on *Arbacia* and *Hipponoe*, it would have been necessary for 25 to 35 per cent of the eggs to have had unfertilized membrane capacities. Unfortunately, there are no

data available on the development of these eggs, but it is believed that the percentages of unfertilized eggs were considerably lower than these figures

On the same basis we calculate from 90 to 100 per cent of fertilized eggs from the present data on suspensions which showed better than 90 per cent fertilization and early development. There is not a complete agreement between the two sets of values as is illustrated by the suspension of Fig. 3, which showed better than 99 per cent development in the 16 cell stage. The impedance locus shows the largest intermediate frequency effect which was found in the present work, corresponding to only 90 per cent with fertilized membrane capacities. We are then left with the possibility that a few eggs may show normal early development but still retain the "unfertilized" membrane capacity after fertilization.

It is difficult to interpret either the absolute values or the change of the internal resistance on fertilization until it is possible to make measurements at considerably higher frequencies than have been used in this work. There is an indication that the highest frequency phenomenon is not dependent upon the integrity of the cell.

It should be pointed out that the  $90^\circ$  phase angle which is found at lower frequencies means that there is no wide variability of membrane capacities among the eggs from a single female. It is found that apparent phase angles less than  $90^\circ$  can be obtained when there is a statistical distribution of the product  $C_m\omega$  among the eggs in the suspension (Cole and Curtis, 1936). We may conclude that the statistical variation of membrane capacities is no greater than the variation of diameters of the eggs from a single female.

There is as yet no certain explanation for the low phase angles which were first found in this work. It may be that these are characteristic of early season eggs, but it seems more likely that they are a result of injury. In either case it is not possible to say for certain either that every egg had a membrane phase angle less than  $90^\circ$ , or that every egg had a  $90^\circ$  phase angle, while the membrane capacities and diameters varied from one egg to another so that the apparent phase angle of the "average" egg was less than  $90^\circ$ . The fact that single eggs with phase angles less than  $90^\circ$  have been measured (Cole and Curtis, 1938) argues against the latter explanation. If injury does cause the

membrane phase angle to fall below  $90^\circ$ , the results in the first *Arbacia* paper (Cole, 1928*b*) may in part be explained as the result of injury due to stirring the suspensions by bubbling

#### SUMMARY

From the low frequency alternating current impedance and the volume concentrations of suspensions of *Arbacia* eggs, it is shown that the high resistance membrane is either at or very near the plasma membrane for both unfertilized and fertilized eggs, and that the specific resistances of the perivitelline space and fertilization membrane are not greatly different from that of sea water. The effect of the capacity element which appears after fertilization at intermediate frequencies is considerably less than in the earlier experiments on *Arbacia* and *Hippoonoe* eggs.

These findings indicate that the fertilization membrane does not have the high capacity previously attributed to it and that the increase in membrane capacity takes place at or near the plasma membrane.

We are very much indebted to Dr. H. J. Curtis for his assistance in this work.

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# ELECTRIC IMPEDANCE OF SINGLE MARINE EGGS\*

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## INTRODUCTION

It was first shown by Fricke (1925) that it is possible to calculate the electrical capacity of the membrane and the resistance of the interior of the red blood cell from impedance measurements made on suspensions of the cells. Since then these properties have been determined for many different types of cells with constantly improved methods of handling the suspensions, of measuring their impedance, and of interpreting the results. Although it is now possible to work with as little as 0.1 cc., the use of suspensions has definite limitations.

In its simplest form the analysis of the paths of the current flow, which forms the basis of the technique of interpretation, requires that the cells of the suspension shall be uniformly distributed, and shall all be identical spheres with identical electrical properties. As the theory becomes more complicated, it is possible to deal with cells having spheroidal form and, less satisfactorily, with suspensions in which the capacity and resistance vary from cell to cell. Also, the interpretation should not be expected to be valid above a 60 per cent volume concentration of cells in the suspension, but, on the other hand, the measurements become rather difficult when the volume concentration is less than 10 per cent. There are many cases where it is difficult or impossible to obtain 0.02 cc. of identical cells and to maintain them in a uniform 20 per cent suspension. For them a technique requiring only a single cell would be very valuable, and although a few of the large unicellular plants have been measured individually, (Blinks, 1936, Curtis and Cole, 1937), the methods used are not easily adaptable to smaller cells.

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Probably the simplest procedure for measuring a single cell is to slide a cell into a snugly fitting capillary tube and measure the impedance between electrodes at the two ends of the tube

### Method

To obtain a satisfactory accuracy of measurement it is only necessary that the resistance of the electrolyte between the electrodes and the cell shall be relatively small, but difficulties are to be expected in the interpretation of the data. A complete theoretical picture of the paths of current flow is more difficult to obtain than in the case of a suspension, partly on account of the geometry of the system and partly because it is not easy to determine the separation between the cell membrane and the capillary wall. However, with the method of measurement and rather crude analysis to be outlined, it has been possible to check the method with single unfertilized and fertilized whole *Arbacia* eggs, and obtain preliminary

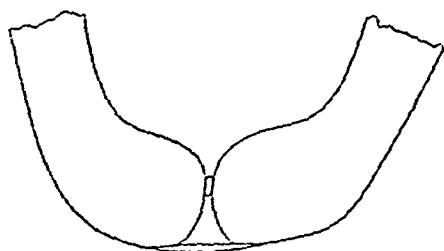


FIG 1 Line tracing made from a photomicrograph of an unfertilized *Arbacia* egg in a  $48\mu$  capillary

data on unfertilized and fertilized white *Arbacia* halves, whole *Cumingia* and *Chaetopterus* eggs, and unfertilized red *Arbacia* halves

The end of a clean 2 or 3 mm thin walled glass tube is heated until it closes down to a short capillary somewhat smaller than the diameter of the egg to be measured. The center portion of such a capillary is usually quite uniform in diameter and, by the proper choice of the initial wall thickness and diameter, almost any desired diameter and length of capillary can be obtained. A flat face is ground and polished on the outside of the tube so that the capillary may be seen without distortion under a microscope.

The outside of the tube is covered with a layer of vaseline to prevent surface losses at high frequencies, filled with sea water, and the capillary end immersed in sea water. An egg is then placed in the tube and when it has settled to the top of the capillary, the outside water level is raised or lowered to push it to the center as shown in Fig 1. The middle portion of the egg is cylindrical and the ends are nearly hemispherical. When the inside and outside pressures are then equalized, the egg will remain in position and in good condition for long periods. Platinized platinum electrodes are then placed in the sea water inside and outside the tube and the impedance between them is measured.

These measurements have been made at frequencies from 1 to 2500 kc (kilocycles per second) with the Wheatstone bridge which has been described by Cole and Curtis (1937). At the end of a frequency run the egg was forced out by raising the pressure, and a sea water run was made. From these data, the series resistance and reactance at each frequency were computed and plotted as the impedance locus.

The method is quite good electrically, although these are errors for frequencies above 2500 kc which prevent the use of higher frequencies. It is often a tedious and tiresome task to get an egg satisfactorily placed in the capillary. Some eggs are quite difficult to handle and, for example, every *Arbacia* red half went into the two cell stage before it could be placed. It is expected that the manipulation can be made more positive and rapid than it is at present.

For the most part the eggs seem to remain in good condition in the capillary. Only one or two cytolized, and a fertilized egg will go into the two and four cell stages in the capillary, although it has not been possible to fertilize an egg in place. Furthermore, several of the *Arbacia* eggs showed 90° phase angles which suggests that they, at least, were quite normal.

#### DATA AND INTERPRETATIONS

##### *Whole Arbacia Eggs*

To investigate the possibilities of this single egg method, the first data were taken on whole unfertilized and fertilized *Arbacia punctulata* eggs whose characteristics have been determined from measurements on suspensions.

The impedance loci, for a single unfertilized *Arbacia* egg, shown in Fig. 2, and for a single fertilized *Arbacia* egg, shown in Fig. 3, have the same form as the loci for suspensions of these eggs when they are found in good condition. These loci show that either egg can be represented over a wide frequency range by equivalent circuits having a single capacity and two or more resistances, such as the two shown in Fig. 4. In both of these  $\bar{R}$ , the resistance of the sea water between the electrodes and the egg, has not been measured directly. It has been eliminated for either circuit by subtracting from the measured resistance of the capillary when filled with sea water, the calculated resistance of a cylinder of sea water having the diameter of the capillary and the volume of the egg. At very high frequencies, where the membrane impedance is negligible, the specific resistance of the egg interior is computed by assuming the egg to have this same cylindrical form. On the other hand, at low frequencies, it will be assumed that

the membrane impedance of the egg is so high that the current density in the exposed hemispherical ends of the membrane is essentially uniform. The effective membrane area at each end will then be that of the hemisphere and the membrane capacity and resistance for a unit area will be computed on this basis.

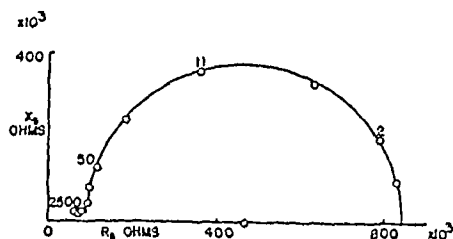


FIG 2

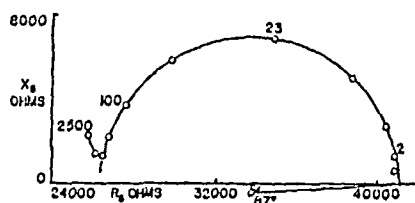


FIG 3

FIG 2 Impedance locus, series resistance,  $R_s$ , vs series reactance,  $X_s$ , for a single unfertilized *Arbacia* egg. Frequencies indicated are in kilocycles per second.

FIG 3 Impedance locus, series resistance,  $R_s$ , vs series reactance,  $X_s$ , for a single fertilized *Arbacia* egg. Frequencies indicated are in kilocycles per second.

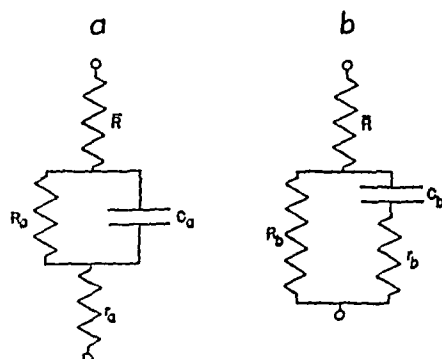


FIG 4 Equivalent circuits for a single egg. (a) Assuming no leakage around the egg. (b) Assuming cell membrane to be non-conducting.

If we now assume that there is no current leakage around the egg, in circuit *a* of Fig 4,  $R_a$  and  $C_a$  represent the resistance and capacity of the two hemispherical end portions of the membrane in series, and  $r_a$  is the resistance of the interior. The computed membrane capacities are 1.4 and 3.15  $\mu\text{f}/\text{cm}^2$  for two unfertilized eggs and 16 and 43  $\mu\text{f}/\text{cm}^2$  for two fertilized eggs, and the membrane resistances are 1.3 and 13  $\text{ohm cm}^2$  for the unfertilized and 0.17 and 0.41  $\text{ohm cm}^2$



the *Cumingia* and *Chaetopterus* eggs were obtained by the usual techniques

## DISCUSSION

Without a complete analytical solution for the current flow, it is difficult to determine the errors due to the approximations which have been made. It seems probable that the assumption of the cylindrical form for the estimation of  $\bar{R}$  and the resistance of the interior does not

TABLE II

*Membrane Capacity and Internal Resistance of Various Marine Eggs Computed on the Basis of Circuit b of Fig. 4*

	Membrane capacity		Internal resistance
	$\mu\text{f/cm}^2$	$\phi$	
<i>Arbacia lixles</i>			
Red unfertilized	0.62	90°	14.5 × sea water
" "	1.4	80°	17.6 × " "
" "	2.15	89.5°	29.7 × " "
" "	2.29	90°	31 × " "
White unfertilized	0.64	—	—
" "	0.625	87°	5.0 × " "
" "	0.635	90°	5.0 × " "
" fertilized	2.25	87.5°	5.1 × " "
<i>Cumingia</i>			
Unfertilized	2.68	90°	7.25 × " "
Fertilized	2.05	90°	6.58 × " "
"	2.62	—	—
<i>Chaetopterus</i>			
Unfertilized	1.15	82°	7.8 × " "
Fertilized	1.32	—	—
"	1.08	—	—

involve an error of more than 5 per cent. The use of the hemispherical caps for the calculation of membrane capacity and resistance seems quite permissible when the space between the egg and the glass wall is small, but otherwise it is difficult to justify. Since 90° phase angles are found, it is quite probable both that the current density is uniform over the effective area and that the effective area changes relatively little with frequency. At the present time, probably the most satisfactory justification of these approximations is

the fact that they lead to the previously determined values for the *Arbacia* eggs

The assumption of a high membrane resistance also is more attractive because it leads to reasonable numerical values, and there are other observations which are consistent with it. When an unfertilized egg with some adherent jelly is placed in the capillary, the low frequency resistance is usually quite low. When the egg is run up and down in the capillary several times the resistance becomes progressively higher and it can be seen that the egg comes closer and closer to the capillary wall. Assuming that all the current flows through the membrane, we are forced to the conclusion that such an egg starts with a very low membrane resistance and high membrane capacity, and that the membrane resistance increases and membrane capacity decreases during this process. On the other hand, assuming a non conducting membrane, the membrane capacity remains constant throughout and the calculated separation between the membrane and the capillary wall decreases. Thus the latter assumption not only leads to a picture which is more reasonable, but also agrees with the microscopic observations.

A membrane resistance of 25 ohm cm<sup>2</sup> or less should give a difference between the actual and the non-conducting volume concentration on the suspension measurement of more than 1 per cent. Although the spread of these differences in the preceding paper (Cole and Spencer, 1938) is large, the average is so nearly zero it seems unlikely that any low values of membrane resistance are to be expected. On the other hand, the highest low frequency resistance yet found for a single egg, over 800,000 ohms, would correspond either to a membrane resistance of 13 ohm cm<sup>2</sup> or to a layer of sea water about 0.2  $\mu$  thick between the membrane and its capillary wall. The former possibility thus seems improbable while the second is very difficult to disprove.

Turning to the fertilized eggs, it was always found that in the capillary they had a relatively low resistance, and behaved and looked like an unfertilized egg with considerable jelly. Assuming a non conducting plasma membrane and a completely permeable fertilization membrane, we arrive at acceptable values for the thickness of the perivitelline space, and as shown in Table I, for the membrane capacity and internal resistance.

It is fairly certain, on the basis of these single egg observations that the plasma membrane resistance of both unfertilized and fertilized *Arbacia* eggs is at least considerably greater than the 25 ohm  $\text{cm}^2$  limit justified by the suspension data. It should be understood, however, that the assumption of a completely non-conducting membrane, which has been used in the interpretation of the data, in no way denies an ionic permeability to the membrane. It merely means that at low frequencies for measurements of this particular type on single eggs and for measurements on suspensions, the current flow through the membrane is so small that it is not possible to detect the difference between a sufficiently small ionic permeability and none at all.

Although an equivalent constant phase angle of less than  $90^\circ$  can result from a statistical distribution of static membrane capacities among the cells of a suspension, the occurrence of low phase angles for single eggs demonstrates that a phase angle of less than  $90^\circ$  can be an inherent characteristic of the egg cell membrane.

The data on fertilized eggs are entirely consistent with a complete ionic permeability for the *Arbacia* fertilization membrane and an increased plasma membrane capacity. Comparing the impedance locus for the fertilized egg, shown in Fig. 3, with the corresponding locus in the previous paper (Cole and Spencer, 1938), it will be seen that there is no deviation from the circle at intermediate frequencies as there is on the curve for a suspension. This deviation is discussed at some length there, and it is pointed out that at least part of it could be explained as being due to non-uniformity of the material. If further experiments on single eggs verify this one experiment in showing no deviation at intermediate frequencies, they will lend rather conclusive support to the non-uniformity postulate.

There are sufficient data on the half *Arbacia* eggs to indicate quite definitely that the membrane capacity for the red half is considerably higher, and for the white half somewhat lower than for the whole egg. It also seems that the granules in the red half increase the internal resistance more than does the oil of the white half. It is possible that the cause of the unexplained high frequency effect shown by whole eggs is primarily transferred to the red half on separation, but the highest frequency data are too uncertain to prove this as yet.



The unfertilized *Chaetopterus* egg showed a membrane capacity slightly higher than the average for other forms, and that of *Cumingia* was even higher. In these forms, the apparent equality of the capacities before and after fertilization is of considerable interest, but should be substantiated by further measurements.

#### SUMMARY

Alternating current impedance measurements have been made on several single marine eggs over the frequency range from 1 to 2500 kilocycles per second. The eggs were placed in the center of a short capillary made by heating the end of a 2 mm thin walled glass tube until it nearly closed, and electrodes were placed in the sea water on each side of the egg.

When it is assumed that the membrane conductance is negligible, the membrane capacity and internal resistances of unfertilized and fertilized *Arbacia* eggs agree with the values obtained from suspensions. Preliminary data on centrifugally separated half *Arbacia* eggs, and whole *Cumingia* and *Chaetopterus* eggs are given.

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# FORMATION OF TRYPSIN FROM TRYPSINOGEN BY AN ENZYME PRODUCED BY A MOLD OF THE GENUS *PENICILLIUM*

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An aqueous solution of crystalline trypsinogen on standing changes autocatalytically into active trypsin without the addition of any activator (kinase). Addition of ammonium sulfate or magnesium sulfate to a solution of trypsinogen greatly accelerates the rate of formation of trypsin as does also the addition of enterokinase or active trypsin (1). The "spontaneous" autocatalytic formation of trypsin from trypsinogen, as well as the accelerating action of salts, enterokinase, or active trypsin takes place best in the range of pH 7.0-9.0. Very little if any trypsin forms in an acid medium at a pH less than 4.0 even with the addition of large amounts of enterokinase or active trypsin. Pure trypsin gradually digests itself in a slightly alkaline medium (2), hence the formation of trypsin at pH 7.0-9.0 is generally accompanied by a considerable loss of the trypsin formed. This paper describes the formation of trypsin from crystalline trypsinogen by means of a new trypsinogen kinase produced, as we accidentally discovered, by a mold of the genus *Penicillium*<sup>1</sup>. It differs markedly from enterokinase in that it brings about the transformation of trypsinogen into trypsin most rapidly at pH 2.5-4.0. In this range of pH trypsin is very stable. Hence the formation of trypsin at pH 2.5-4.0 by the new kinase is not accompanied by any measurable autolysis of the trypsin formed.

The action of the mold kinase in the process of transformation of trypsinogen into trypsin is that of a typical enzyme. The process

<sup>1</sup> The presence of a trypsinogen kinase in bacteria and in a few poisonous mushrooms has been reported as early as 1902: see Delezenne, C., *Compt rend Soc biol*, 54, 998, 1902; 55, 27, 1903.

follows the course of a catalytic unimolecular reaction, the rate of formation of trypsin being proportional to the concentration of kinase used. The ultimate amount of trypsin formed, however, is independent of the concentration of kinase used.

Active trypsin produced by means of mold kinase has been crystallized. It appears to be identical in crystalline form with the crystalline trypsin produced autocatalytically from crystalline trypsinogen at pH 8.0. The two products have, within the experimental error, the same solubility and specific activity.

Mold kinase is most conveniently obtained by cultivating the *Penicillium* mold in a synthetic liquid medium of pH about 4.0. The kinase appears in the medium and its concentration increases during the growth of the mold and continues to increase for some time even after the mold has ceased growing, so that there is no constant relation between the concentration of the kinase in the medium and the weight of the mold.

Mold kinase is rapidly destroyed at pH 6.5 or higher and any culture medium of the *Penicillium* organism of pH above 6.5 fails to show the presence of any kinase activity.

Mold kinase is inactivated when heated at temperatures above 50°C even at the pH of its maximum stability. The critical thermal increment for the process of heat inactivation at 50°C and 60°C is  $\mu = 53,500$  calories which is comparable with that of denaturation of proteins (3) and of inactivation of trypsin (4) and enterokinase (5). When heated at 70°C mold kinase is completely inactivated within 5 minutes.

The molecular weight of the mold kinase as determined by diffusion is about 40,000. It does not diffuse through an ordinary collodion membrane.

The high temperature coefficient of inactivation of mold kinase as well as its high molecular weight suggest a possible protein nature for the molecule of mold kinase.

## EXPERIMENTAL

### *I Some Properties of Mold Kinase*

*1 Cultivation of the Penicillium Mold*—The mold was originally isolated from an old non-sterile stock of  $M/400$  hydrochloric acid which

showed slight activating power when mixed with a solution of crystalline trypsinogen and which contained a slight growth of mold on the bottom of the container. The mold was cultivated first on potato agar medium, single colonies of the organism on the plate were repeatedly transferred to fresh medium. Single spore preparations of the organism were finally obtained.<sup>2</sup> The pure culture<sup>2</sup> of the organism was henceforth propagated on a liquid medium consisting of

Sucrose	7.20 gm
Dextrose	3.60 "
MgSO <sub>4</sub> (crystals)	1.23 "
KH <sub>2</sub> PO <sub>4</sub>	13.62 " (0.1 mol)
KNO <sub>3</sub>	2.00 "
H <sub>2</sub> O	1000 ml

The medium was distributed in 250 ml Pyrex Erlenmeyer flasks 100 ml in each flask, and autoclaved for 1/2 hour at 15 pounds steam pressure. 10 ml of concentrated lactic acid was then added to each flask. The flasks were inoculated with 10 ml of a uniform suspension of spores and the mold was allowed to grow at a room temperature of about 20°C.

*2 Estimation of Mold Kinase. The Mold Kinase Unit [M K U] —* The quantity of mold kinase in any sample is expressed in terms of the velocity with which it transforms crystalline trypsinogen into trypsin under standard conditions.

One mold kinase unit, 1 [M K U], is defined as the amount of kinase that brings about the activation of 0.065 mg of crystalline trypsinogen at the rate of unity (100 per cent) per unit of time (1 hour) at pH 3.4 and at 35°C.

The isolation and cultivation of single spores was kindly done for us by Dr Arnold J. Ullstrup of the Department of Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey.

<sup>2</sup> A pure culture of the organism was submitted for identification to Dr Charles Thom of the U. S. Department of Agriculture at Washington, D. C. Dr Thom stated that the organism is a member of the genus *Penicillium* belonging to the very questionable group designated as *lanata-divaricata* with characteristics running all the way from *simplicissimum* through *janthnellum* to *soppi*, the exact strain being very difficult to determine.<sup>1</sup>

Dr Selman A. Waksman of the State of New Jersey Agricultural Experiment Station kindly tested the organism for production of citric acid, which he found to be negative.

The rational derivation of the definition of the unit is discussed in the section of this paper which deals with the kinetics of formation of trypsin, where a standard method is also described for the determination of the number of [M K U] in a sample of mold kinase. The standard method involves a large number of measurements. For practical purposes the following simplified procedure was developed.

Activation mixture

1.0 ml sample of kinase, plus

3.5 ml  $\text{M}/10$  citrate buffer pH 3.4, plus

0.5 ml of stock crystalline trypsinogen in  $\text{M}/200$  HCl containing 0.65 mg protein per ml (0.1 mg protein nitrogen per ml)

The activation mixture is placed for 30 minutes in a water bath at  $35^{\circ}\text{C}$ . 1.0 ml of the mixture is then added to 5.0 ml urea-hemoglobin and its active trypsin content,  $[\text{T U}]^{\text{Hb}}$ , is determined as described by Anson and Mirsky (6). The number of mold kinase units [M K U] per ml activation mixture corresponding to the number of  $[\text{T U}]^{\text{Hb}}$  measured is then read off a standard curve. The standard curve is obtained by plotting the data of  $[\text{T U}]^{\text{Hb}}$  vs [M K U] for a series of activation mixtures containing various dilutions of a stock of mold kinase of known [M K U] content, as determined by the standard method.

3 *The Rate of Accumulation of Mold Kinase in the Penicillium Culture Medium*  
—A series of 250 ml Pyrex Erlenmeyer flasks, each containing 100 ml of sterilized liquid medium, was inoculated with 1.0 ml of a uniform suspension of *Penicillium* spores and kept at  $20^{\circ}\text{C}$ . At definite intervals of time, the contents of individual flasks were filtered through dried and weighed Whatman No. 42-9 cm filter paper. The filtrate in each case was used for analysis for its kinase and phosphate content, while the mold residue on the paper was washed several times with distilled water and then dried on the filter paper for 24 hours at  $100^{\circ}\text{C}$  and weighed. The phosphate values were used as a basis to correct the data for the concentration of the kinase per milliliter of medium for losses in volume due to evaporation. The pH of the medium rises gradually during the later stage of the growth of the mold even in the presence of  $\text{M}/10$   $\text{KH}_2\text{PO}_4$ . Hence concentrated lactic acid in doses of 1.0 ml per flask was added whenever indicator tests showed that the pH had risen above 5.6.

Fig. 1 shows that the curve for the growth of the *Penicillium* organism as well as the curve for the accumulation of kinase are of the form typical for growth of microorganisms in a limited amount of nutritive medium. The accumulation of kinase in the medium proceeds initially at a very slow rate as compared with the rate of growth of the mold, but it becomes very rapid at the time when the mold reaches its final growth. The maximum rate of increase of the concentration

of the kinase in the medium coincides approximately with the time of spore formation by the mold. The concentration of kinase in the medium continues to increase for some time even after the mold has stopped growing. Thus there is no constant relation between the rate of growth of the organism and the rate of accumulation of kinase in the medium. A similar observation was made recently by Scribner and Krueger (7) for the rate of growth of phage and bacteria, namely that under certain conditions there is a continuous increase in the concentration of phage in the medium for some time after the bacteria have ceased to multiply.

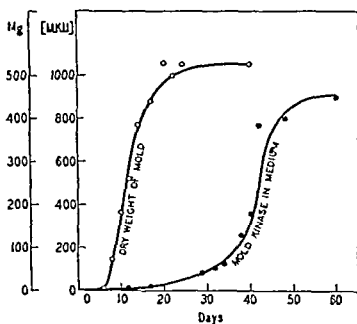


FIG. 1 Rate of formation of trypsinogen kinase in the culture medium of *Penicillium*

The rate of enzyme formation in cultures of microorganisms depends on experimental conditions and may be either proportional to or independent of the increase in the number of organisms.<sup>4</sup>

- 4 *Stability of Mold Kinase at Various pH*—Mixtures were made of  
 1.0 ml. kinase containing 500 [M K U] plus  
 1.0 ml. of  $\pi/10$  buffer solutions of various pH, plus  
 3.0 ml.  $H_2O$ . Left at 25°C

Samples were measured for pH and kinase content immediately after mixing and again after 1.5 hours

<sup>4</sup> Cf. Yudkin, J. *Biol. Rev. Cambridge Phil. Soc.* 1938, 13, 93

The results are given in Fig 2 which shows that mold kinase is quite stable between pH 2.0 and 6.0 and is very rapidly destroyed at pH 7.0. Mold kinase thus differs from enterokinase which has an optimum stability in a region extending from pH 6.0–8.0 (8).

5 *Inactivation of Mold Kinase by Heat*—Mold kinase is completely inactivated within 5 minutes when heated at 70°C. At lower temperatures the inactivation proceeds at a measurable rate. Fig 3 shows the curves for inactivation at 50°C and 60°C of dialyzed mold kinase made up in M/50  $\text{KH}_2\text{PO}_4$  and containing about 6 [M K U] per

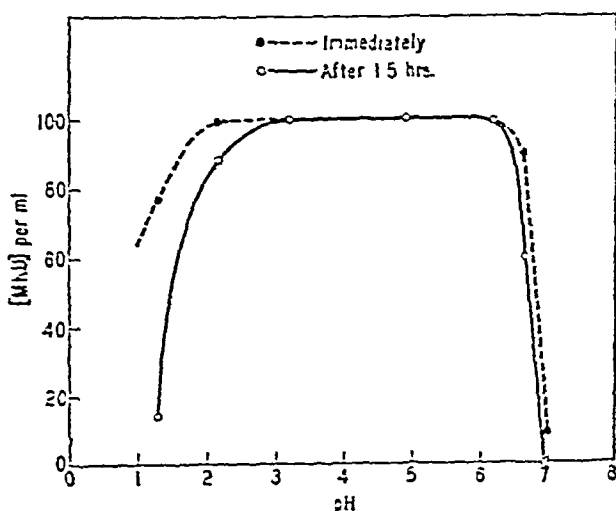


FIG 2 Stability of mold kinase at various pH

ml. The inactivation at both temperatures evidently follows a unimolecular course in accordance with the equation

$$-\frac{dM}{dt} = KM \quad (1)$$

which when integrated gives

$$\ln \frac{M_0}{M} = Kt \quad (2)$$

$M_0$  = initial concentration of kinase

$M$  = concentration of kinase at any time  $t$

$K$  = velocity constant of inactivation



$K$  is the slope of the straight line obtained when values for  $\ln \frac{M_0}{M}$  are plotted against corresponding  $t$  values. The curves show that the velocity constants for inactivation of mold kinase pH 4.0 at 50°C and 60°C are

$$K_{50} = 0.0073 \text{ per minute}$$

$$K_{60} = 0.088 \text{ per minute}$$

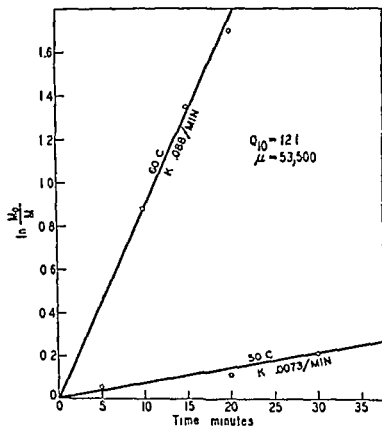


FIG. 3 Rate of inactivation of mold kinase at 50°C and 60°C  $Q_{10} = 12.1$   
 $\mu = 53,500$  calories per mol

with a temperature coefficient of  $Q_{10} = 12.1$

Substituting  $\ln \frac{K_{60}}{K_{50}}$  in Arrhenius equation

$$\ln \frac{K_2}{K_1} = \frac{\mu(T_2 - T_1)}{2T_1T_2} \quad (3)$$

we get a value for "the critical thermal increment" of inactivation of mold kinase

$$\mu_{60-50} = 53,500 \text{ calories per mol}$$

which is of the same high order as that obtained for denaturation of proteins and of inactivation of enzymes

*6 Chemical Nature of Mold Kinase Molecular Volume*—We have not made as yet any serious attempt to isolate the mold kinase in pure form. Preliminary studies showed that the kinase can be purified to some degree by dialysis and fractional precipitation with ammonium sulfate. It is non-volatile and can be concentrated by vacuum distillation at about 35°C.

The rapid inactivation of mold kinase when heated to 70°C as well as the high temperature coefficient of inactivation suggest a possible protein nature for the molecule of mold kinase which is in agreement with its high molecular volume of 31,000 cm<sup>3</sup> and molecular weight of 41,000 gm (assuming a density of 1.3) as determined by the diffusion method of Northrop and Anson (9). The value of the diffusion coefficient is

$$D = 0.05 \text{ cm}^2/\text{day at } 10^\circ\text{C}$$

## II Formation of Trypsin from Crystalline Trypsinogen by Mold Kinase

*1 Effect of pH on Rate of Formation*—Activation mixtures were made consisting of

1.0 ml mold kinase solution containing 50 [M K U] per ml, plus

3.5 ml M/10 citrate buffer of various pH, plus

0.5 ml of 0.065 per cent solution of crystalline trypsinogen in M/200 HCl

Mixtures were allowed to stand for 30 minutes at 35°C. The amount of formed trypsin in each mixture was then determined by the hemoglobin method.

The results are given in Fig. 4 which shows that mold kinase, unlike enterokinase, transforms trypsinogen into trypsin in an acid medium only, the range of pH favorable for the reaction extending approximately from 2.0–4.5 with a region of optimum rate at about pH 3.4.

*2 Effect of Varying the Concentration of Mold Kinase on the Extent of Formation of Trypsin from Trypsinogen*—A series of activation mixtures was made each one containing

1.0 ml of stock of 0.065 per cent of crystalline trypsinogen, plus

8.0 ml of M/10 citrate buffer pH 3.4, plus

1.0 ml of various concentrations of mold kinase made up in M/10 citrate buffer pH 3.4, the concentrations varying from 0.75 — 7.5 [M K U] per ml

The mixtures were left at 4°C. Samples of 1.0 ml. of each mixture were tested at various times for tryptic activity

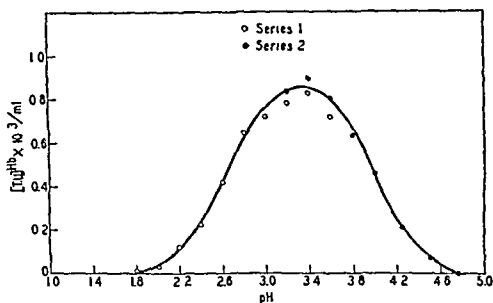


FIG. 4 Effect of pH on rate of activation of crystalline trypsinogen by mold kinase

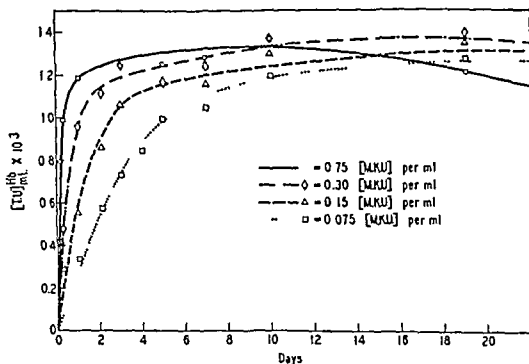


FIG. 5 Effect of kinase concentration on extent of formation of trypsin from crystalline trypsinogen at pH 3.4 and 5°C

Fig. 5 gives the results of the measurements. It is evident that the rate of formation of trypsin increases with the increase in concentra-

tion of kinase used, but the ultimate amount of trypsin formed is independent of the concentration of kinase added. The same series was repeated with a concentration of trypsinogen 20 times as high as that used in the first series. The result appeared to be the same, thus proving that the kinase in its action resembles a typical catalyst.

3 *Is Formation of Trypsin Accompanied by Loss of Protein?*—It had been observed that during the process of autocatalytic formation of trypsin from crystalline trypsinogen at pH 7.6 there is a gradual loss of protein as measured by the amount of precipitate formed on addition of trichloroacetic acid. This loss may be due either to autolysis of the trypsin formed, which is known to take place readily at pH 7.0 or higher, or to a possible cleavage of the trypsinogen molecule during the reaction. The change of trypsinogen into trypsin by mold kinase at pH 3.4 is accomplished under conditions where no autolysis of the trypsin formed can take place,<sup>5</sup> and any possible loss of protein in the activation mixture consequently could be attributed to a partial hydrolysis of the trypsinogen molecule during the process of activation.

A preliminary experiment showed, however, that there is no measurable loss of protein when crystalline trypsinogen is activated by mold kinase at pH 3.4 and 5°C.

4 *Kinetics of Formation of Trypsin* (a) *Effect of Varying Concentration of Mold Kinase on the Rate of Reaction*—The formation of trypsin from crystalline trypsinogen by mold kinase at pH 3.4 is not accompanied by any autocatalytic formation of trypsin. This is due to the fact that trypsin is inactive at this pH. The time rate of formation of trypsin by the mold kinase follows the course of a catalytic unimolecular reaction, and is expressed by the differential equation

$$\frac{dA}{dt} = KM(A_0 - A) \quad (4)$$

where  $K$  equals velocity constant,  $M$  is the initial concentration of kinase in the activation mixture which, like any other catalyst, re-

<sup>5</sup> A slight hydrolysis of the trypsin formed takes place if activation proceeds at 35°C or higher due to the action of a very weak proteolytic enzyme which was found to be associated with the mold kinase, like kinase the optimum pH range or the proteolytic activity is between 3.0–4.0. Its effect on the formed trypsin becomes negligible if activation is accomplished at a temperature of 5–10°C.

mains unchanged during activation,  $A$  is the concentration of trypsin in the activation mixture at any time  $t$ , and  $A_0$  is the final concentration of trypsin when the activation is complete ( $A_0 - A =$  concentration of trypsinogen at any time  $t$ ) When integrated equation 4 becomes

$$\ln \frac{A_0}{A_0 - A} = (KM)t \quad (5)$$

$(KM)$  being the slope of the straight line obtained when the values of  $\ln \frac{A_0}{A_0 - A}$  are plotted against the corresponding values of  $t$

Fig 6 shows the experimental time rate curves for the activation at 35°C of a definite amount of crystalline trypsinogen by two concentrations of mold kinase in the ratio of 1.0 to 2.5 The experimental points in each case are on a straight line except for the slight deviation of the last few points due to the action of the proteolytic enzyme associated with the kinase (see footnote 5) The slopes of the straight lines are proportional to the concentrations of kinase used, namely

$$KM_1 = 0.013 \text{ per minute}$$

$$KM_2 = 0.0325 \quad ' \quad '$$

and

$$\frac{KM_1}{KM_2} = 2.5$$

(b) *Effect of Varying the Concentration of Trypsinogen on the Velocity Constant of Formation of Trypsin*—Equation 5 predicts that for any given concentration of kinase the velocity constant  $K$  should be independent of the magnitude of the initial concentration of trypsinogen used Actually, this prediction does not hold true as has been found to be the case with a large number of other enzymes such as urease (10), trypsin (11), invertase (12), etc

Fig 7 shows the experimental curves for the rate of activation of two different amounts of crystalline trypsinogen by mold kinase at pH 3.4 and 6°C, the concentration of mold kinase used being identical in both cases There is a decrease in the velocity constant as the

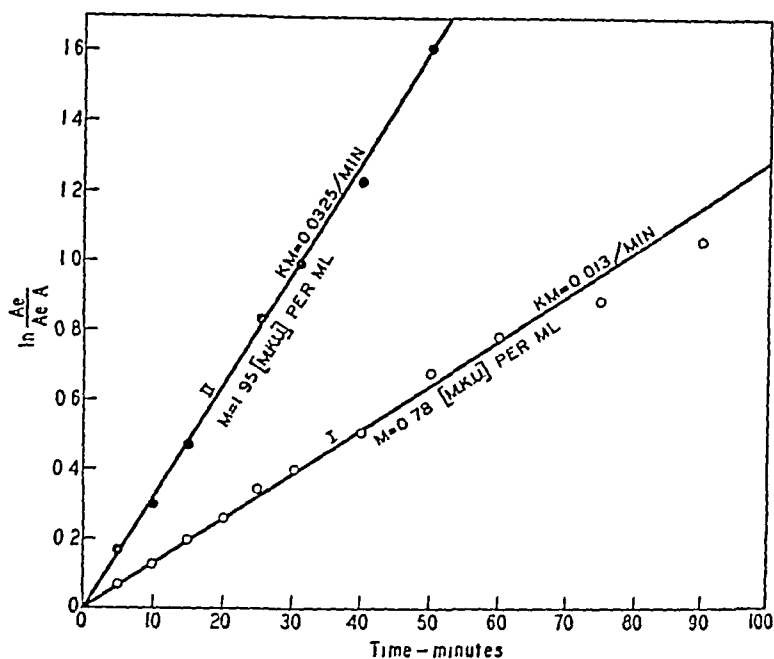


FIG 6 Rate of formation of trypsin from crystalline trypsinogen by mold kinase Concentration of trypsinogen = 0.01 mg protein nitrogen per ml

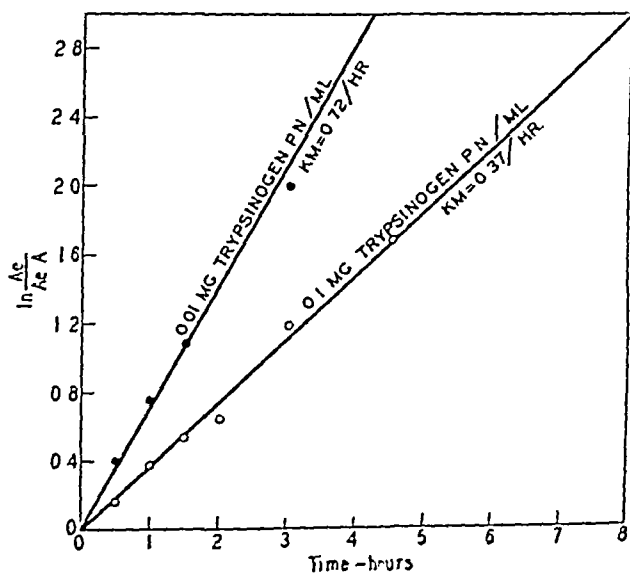


FIG 7 Effect of concentration of trypsinogen on the velocity constant of the formation of trypsin by mold kinase

concentration of trypsinogen used is increased. The velocity constant is reduced 50 per cent for a tenfold increase in initial concentration of trypsinogen.

Activation mixtures used

14.0 ml.  $M/10$  citrate buffer pH 3.4, plus

4.0 ml. solution of mold kinase containing 15 (M.K.U.) per ml., plus

2.0 ml. solution of crystalline trypsinogen in  $M/200$  HCl containing either 1.0 or 0.1 mg. protein nitrogen per ml.

Mixed cold and left at 6°C. Samples tested for tryptic activity at various intervals of time.

(c) *The Equation for the Kinetics of Formation of Trypsin As a Basis for the Definition of the Mold Kinase Unit [M.K.U.]*—The numerical value of the velocity constant  $K$  for the rate of formation of trypsin from crystalline trypsinogen by mold kinase as given in equation 4 depends on the value of the unit chosen to express the magnitude of the kinase concentration  $M$ .  $K$  can be made equal to unity by choosing the appropriate unit for  $M$ . Writing equation 4 in the form of

$$-\frac{dG}{Gdt} = KM$$

where  $G$  is the concentration of trypsinogen at any time  $t$  and putting  $K = 1$ , we get

$$-\frac{dG}{Gdt} = M$$

$M$  equals unity when

$$-\frac{dG}{Gdt} = 1$$

i.e., when the activation proceeds at the rate of 100 per cent per unit of time (under specified conditions of temperature, pH, and initial concentration of trypsinogen). The standard activation mixture used in our studies consisted of the following

1.0 ml. of a solution of crystalline trypsinogen in  $M/200$  HCl containing 0.1 mg. protein nitrogen per ml., plus

2.0 ml. of solution of kinase pH 3.4, plus

7.0 ml. of  $M/10$  citrate buffer pH 3.4

Activation was allowed to proceed at 35°C. The hour was taken as the unit of time. The definition of the mold kinase unit under these conditions is as follows. One mold kinase unit, 1 [M K U], is the amount of kinase that brings about activation of 0.065 mg crystalline trypsinogen (0.01 mg protein nitrogen) at pH 3.4 and 35°C at the rate of 100 per cent per hour.

The standard method of determining the concentration of kinase in the activation mixture in [M K U] per ml consists in measuring the tryptic activity of the mixture at various intervals and then plotting the values of  $\ln \frac{A_0}{A_0 - A}$  against time. The slope of the straight line drawn through the plotted points gives the concentration of kinase in [M K U] per ml of activation mixture. Thus in Fig. 6, where the data plotted have been obtained under the standard conditions mentioned before, the slope of line I is 0.013 per minute or 0.78 per hour, and the slope of II is 0.0325 per minute or 1.95 per hour, the concentrations of mold kinase in the activation mixtures are then 0.78 [M K U] per ml and 1.95 [M K U] per ml correspondingly. It is evident that this method of measuring kinase activity involves the determination of tryptic activity of several samples. As a general routine, only the 30 minute sample is measured and the concentration of kinase corresponding to the found value of  $A$  is read off a standard curve described before. It can also be calculated by substituting  $A$  in the equation

$$\ln \frac{A_0}{A_0 - A} = 0.5 M$$

The value of  $A_0$  is readily determinable for any stock of trypsinogen by using an excess of kinase in the activation mixture.

(d) *Effect of Temperature on the Rate of Formation of Trypsin* — Fig. 8 shows the curves for the rate of formation of trypsin from crystalline trypsinogen by mold kinase at various temperatures. The activation mixtures used were of the standard type containing 0.01 mg trypsinogen protein nitrogen and 0.75 [M K U] per ml. The plotted curves for  $\ln \frac{A_0}{A_0 - A}$  are all straight lines. The velocity constants were obtained by dividing the values for the slopes of the lines by the value of  $M = 0.75$ . When plotted against the reciprocals of the corresponding absolute temperatures the values of  $\ln K$  do not



fall in a straight line, as shown in Fig 9. The calculated temperature coefficients  $Q_{10}$  as well as the values for  $\mu$  are given in Table I. The temperature coefficient  $Q_{10}$  for the range of 25–35°C is much lower

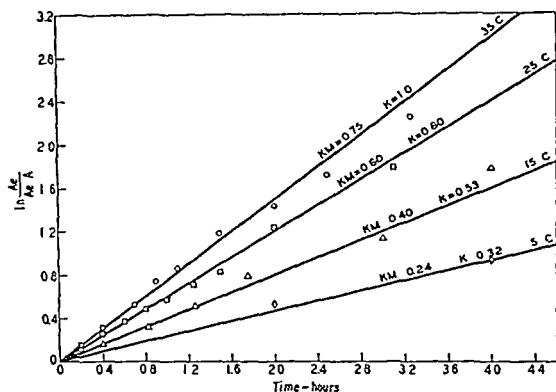


FIG 8 Effect of temperature on the rate of formation of trypsin by mold kinase

TABLE I  
Temperature Coefficient for the Rate of Formation of Trypsin

Temperature	K	$Q_{10}$	$\mu$
C	per hour		calories per mol
5	0.32		
5–15		1.66	8.100
15	0.53		
15–25		1.51	7.000
25	0.80		
25–35		1.25	4.250
35	1.00		

than the temperature coefficient of about 1.8 found for the rate of digestion of hemoglobin by pepsin (13) or trypsin (14).

The values of  $\mu$  obtained here for the formation of trypsin compare

in magnitude with those obtained for hydrolysis of ethyl butyrate by pancreatic lipase (15) as well as for the decomposition of hydrogen peroxide by liver catalase (16)

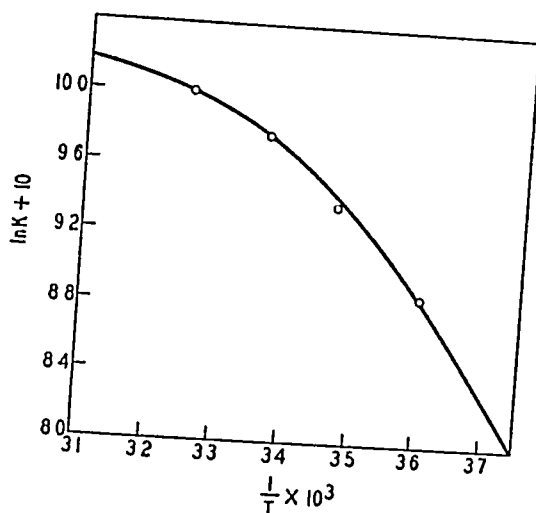


FIG 9 Velocity constant of activation vs reciprocal of absolute temperature

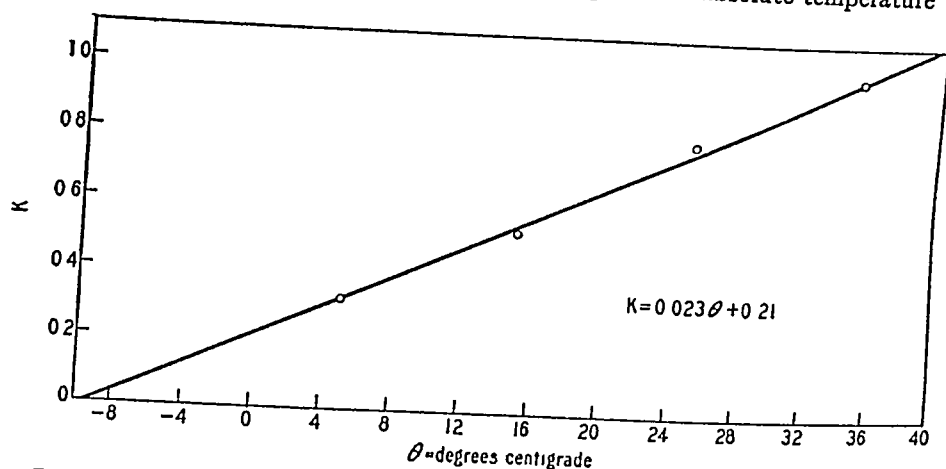


FIG 10 Linear relation between temperature and velocity constant of activation

It is to be observed that when the velocity constants for the rate of activation of crystalline trypsinogen by mold kinase are plotted against temperature directly they fall in a straight line (Fig 10) the equation of which is

$$K = 0.023\theta + 0.21$$

where  $\theta$  = temperature in degrees centigrade. The velocity constant thus appears to increase by a definite amount of 0.023 for a one degree rise in temperature. Equation 6 can be readily changed into the convenient formula

$$K = K_0(1 + 0.11\theta)$$

where  $K_0$  is the value of  $K$  at  $0^\circ\text{C} = 0.21$

*5 Preparation of Crystalline Trypsin from Trypsinogen by Mold Kinase*—30 ml of dialyzed crystalline trypsinogen containing 10 mg protein nitrogen per ml were mixed with 120 ml  $M/10$  citrate buffer pH 3.4 and 6 ml of solution of mold kinase containing 2,000 [M K U] per ml. The mixture was left at  $5^\circ\text{C}$  for 18 hours. Solid ammonium sulfate was then added so as to bring the solution to 0.7 saturation. The precipitate formed was filtered with suction and washed with saturated magnesium sulfate in  $N/50$  sulfuric acid at room temperature. Filter cake (7.5 gm) was dissolved in 7.5 ml ice cold  $0.4 M$  borate buffer pH 9.0 and 7.5 ml saturated magnesium sulfate added. Solution was left at  $5^\circ\text{C}$ . Crystals of typical trypsin needles began to appear rapidly, and the crystallization was complete in a few hours. The crystals were filtered after 24 hours and the yield was 3 gm of crystal cake of a specific activity of 0.19 [T U] $_{\text{mg P.N.}}^{\text{Hb}}$  which is the same as found for the purest trypsin crystals formed from crystalline trypsinogen by autocatalysis (17).

The solubility of the new crystalline trypsin in saturated magnesium sulfate at  $10^\circ\text{C}$  is the same as that of the trypsin formed from trypsinogen by autocatalysis and a solution saturated with the crystals of either one of the trypsin preparations does not dissolve any crystals of the other kind, thus indicating the identity of the two preparations. The new preparation of the crystalline trypsin gives a solubility curve with saturated magnesium sulfate typical of a pure substance, as shown in Fig. 11. Since the trypsin formed from trypsinogen by the mold kinase is the same as that formed by trypsin the mold kinase must cause the same change in the molecule of trypsinogen as does trypsin.

### *III Activation of Chymo Trypsinogen by Penicillium Kinase*

In addition to its powerful activating effect on trypsinogen mold kinase appears to have a slight activating effect on chymo trypsinogen. The rate of activation of chymo trypsinogen is only about 2 per cent of that of trypsinogen. Preliminary experiments indicate that the

optimum pH range for activation of chymo-trypsinogen by the mold kinase is the same as for activation of trypsinogen. The pH range of stability of the kinase is also the same with respect to activation of either chymo-trypsinogen or trypsinogen. It thus appears that both kinase activities are associated with one and the same substance.

### Methods

1 *Preparation of Crystalline Trypsinogen*—Method of Kunitz and Northrop (18)

2 *Protein Nitrogen by Turbidity*—5 ml of sample are mixed in 150 × 15 mm test tube with 5 ml 5 per cent trichloroacetic acid made up in 0.25 saturated ammonium sulfate. The mixture is allowed to stand at least 5 minutes at 20°C and then placed for 10 minutes in a water bath at 85°C. The test tube is immersed

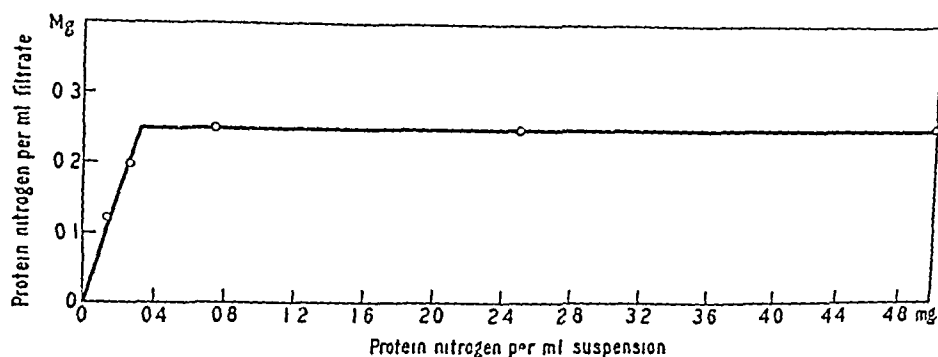


FIG. 11 Solubility of crystalline trypsin in saturated magnesium sulfate pH 4.0 at 10°C in the presence of increasing quantities of solid phase

in the hot water to a depth about 2.0–5.00 mm above the level of the liquid inside the tube and is loosely stoppered during the heating. The tube is stoppered tightly immediately after removal from the bath and left to cool to room temperature. The turbidity of the well mixed suspension is determined in a Klett photoelectric colorimeter (19) against a suspension of the same protein material of a known protein nitrogen concentration as determined by Kjeldahl analysis. A more convenient way is to draw a calibration curve for the turbidity of several concentrations of the standard solution as read against a glass disk or a solution of  $M/25$  copper sulfate in  $M/10$  sulfuric acid. This avoids the necessity of preparing fresh standards. The range is from 0.001–0.005 mg protein nitrogen per ml suspension.

3 *Tryptic Activity Determinations*—The tryptic activity was measured by the rate of digestion of hemoglobin as described by Anson and Mirsky (6) except that the suspension after the addition of trichloroacetic acid was allowed to stand 1/2 hour before filtering. 2.0 ml of 1 per cent of merthiolate in 1.4 per cent borax

were added to each liter of stock of hemoglobin solution, as a preservative, instead of toluene. The latter has been found to interfere with the measurement of tryptic activity.

4 *Phosphorus Determination*—The colorimetric method of Fiske and Subbarow (20)

#### SUMMARY

1 A powerful kinase which changes trypsinogen to trypsin was found to be present in the synthetic liquid culture medium of a mold of the genus *Penicillium*.

2 The concentration of kinase in the medium is increased gradually during the growth of the mold organism and continues to increase for some time even after the mold has ceased growing.

3 Mold kinase transforms trypsinogen to trypsin only in an acid medium. It differs thus from enterokinase and trypsin which activate trypsinogen best in a slightly alkaline medium.

4 The action of the mold kinase in the process of transformation of trypsinogen is that of a typical enzyme. The process follows the course of a catalytic unimolecular reaction, the rate of formation of a definite amount of trypsin being proportional to the concentration of kinase added. The ultimate amount of trypsin formed, however, is independent of the concentration of kinase used.

5 The formation of trypsin from trypsinogen by mold kinase is not accompanied by any measurable loss of protein.

6 The temperature coefficient of formation of trypsin from trypsinogen by mold kinase varies from  $Q_{5-15} = 1.70$  to  $Q_{25-30} = 1.25$  with a corresponding variation in the value of  $\mu$  from 8100 to 4250.

7 Trypsin formed from trypsinogen by means of mold kinase is identical in crystalline form with the crystalline trypsin obtained by spontaneous autocatalytic activation of trypsinogen at pH 8.0. The two products have within the experimental error the same solubility and specific activity. A solution saturated with the crystals of either one of the trypsin preparations does not show any increase in protein concentration or activity when crystals of the other trypsin preparation are added.

8 The *Penicillium* mold kinase has a slight activating effect on chymo trypsinogen the rate being only 1–2 per cent of that of trypsinogen. The activation, as in the case of trypsinogen, takes place only in an acid medium.

9 Mold kinase is rapidly destroyed when brought to pH 6.5 or higher, and also when heated to 70°C. In the temperature range of 50–60°C the inactivation of kinase follows a unimolecular course with a temperature coefficient of  $Q_{10} = 12.1$  and  $\mu = 53,500$ . The molecular weight of mold kinase, as determined by diffusion, is 40,000.

The writer was assisted in this work by Margaret R. McDonald.

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# THE MEASUREMENT OF SURFACE FILMS FORMED BY HEMOCYANIN, TOBACCO MOSAIC VIRUS, VACCINIA, AND BACTERIUM GALLINARUM

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The studies of Gorter (1), Hughes and Rideal (2), Neurath (3), and Langmuir (4) have shown that proteins are capable of forming surface films of a thickness and area independent of the diameter of the molecule. Depending on the pH and salt concentration of the solution upon which the protein is spread, the surface area may be more than 5 times greater than that to be expected from a monolayer of contiguous spherical molecules. The thickness of such surface films is correspondingly less than the assumed spherical molecular diameter. The proteins so far examined by Gorter develop their maximum spreading at pH 1, and again at the isoelectric point, 1 mg covering about 1 sq meter. The same maximum spread may be induced at any pH by the addition of salts (5). This remarkably constant area has been demonstrated for pepsin, trypsin, ovalbumin, and insulin, all having a molecular weight of about 35,000, as well as for zein (6) having a molecular weight 6 times greater.

Recently, Philippi (7) has reported film measurements on some of the high molecular weight respiratory proteins. He has found that one of these, derived from *Palmaris vulgaris*, spread to the same extent as egg albumin at pH 1, but did not spread on solutions of a higher pH. He points out the fact that at pH 1 the molecule of this protein is broken down into smaller components to which he attributes the spreading.

It should be noted that proteins which form films of 1 sq meter per mg at pH 1 and at their isoelectric point develop smaller films of the same thickness at other hydrogen ion concentrations. The area of the film is conditioned by the amount which remains on the surface,

since the thickness of the film appears to be invariable. If a film of egg albumin occupying 0.1 sq meter per mg at pH 3 is brought to pH 1, it will not expand to 1.0 sq meter per mg, but will retain its original area.

An indirect confirmation of this fact was obtained by determining the amount of pepsin entering a subsurface solution at pH 4.6. According to Gorter, pepsin forms a film of only 0.1 sq meter per mg at this pH. One would then expect to recover 90 per cent of the pepsin from the solution under the film. Actually, from 70 to 90 per cent was demonstrated under a film of almost exactly 0.1 sq meter per mg (0.2 mg = 210 sq cm). However, the accuracy of activity measurements with such necessarily dilute solutions ( $4 \times 10^{-7}$  gm protein per cc) is not great.

The results to be presented deal with film area measurements applied to *Limulus* hemocyanin and tobacco mosaic virus. In addition, the surface behavior of protein-containing particles, namely vaccinia virus and a Gram-negative non-motile bacillus, was examined.

### *Materials and Methods*

The Langmuir tray (8) was used to measure surface areas. Certain minor modifications were introduced. Instead of air jets to prevent leakage of the film around the ends of the movable barrier, paraffined threads were attached to the edges of the tray and to the movable barrier. Solid sticks of high melting point paraffin were used to push the film toward the movable barrier. A strip of thin celluloid for the movable barrier was found to be more durable than paraffined paper and equally satisfactory. Each film area was measured not longer than 30 seconds after the protein was applied to the surface, at pressures ranging from 0 to 60 dynes per cm. The areas given in the accompanying curves represent the areas of the films at 0 dynes per cm. The pH of the tray solutions was determined electrometrically by means of the quinhydrone electrode. The pH from 1 to 3.1 was obtained with HCl. From pH 3.3 to 5.2, 0.02 M acetate buffers were used, and from pH 6.0 to 7.9, 0.02 M phosphate buffers were used. The protein solutions were applied to the surface by means of a platinum loop delivering 0.006 cc. Entirely reproducible results were obtained by this method, which was simpler than the micro-pipette employed by Gorter.

The proteins to be tested were obtained in as pure a state as possible. A sample of 3 times crystallized, dialyzed swine pepsin was made available by Dr. R. M. Herriott. The ovalbumin was also crystallized 3 times and dialyzed free of ammonium sulfate. Hemocyanin (molecular weight, 3 million) (9) was obtained from the blood of *Limulus polyphemus* and purified in the quantity ultracentrifuge by a method previously described (10). A sample of ultracentrifuge-purified



tobacco mosaic virus protein (molecular weight, 17 million) (11) was kindly supplied by Dr H S Loring. Vaccinia elementary bodies were prepared by the method described by Craigie (12), which yielded a final product showing practically nothing but Paschen bodies when examined by the Morosow stain. The isoelectric point was found by cataphoresis measurements to be pH 5.0. The suspension of *B. gallinarum* (fowl typhoid) was made from a 5 hour culture in digest broth which had been passed through a Berkefeld N filter to remove larger particulate matter before inoculation. The organisms were sedimented and resuspended 4 times in pH 7.6 buffered saline. The isoelectric point was determined by cataphoresis to be pH 4.1.

The surface measurements on the bacteria and elementary bodies were made the same day they were prepared or within 12 hours. The hemocyanin was stored 3 days in a concentrated solution and diluted just before use. On the basis of Kjeldahl nitrogen estimations all of the preparations were adjusted to a concentration of 2.5 mg. of protein per cc.

### RESULTS

The relation of pH to spreading on 0.02 M solutions is shown in Figs 1 and 2. Tray solutions adjusted to the isoelectric point of each of the materials to be tested were included in the series. Pepsin and ovalbumin developed surface areas in agreement with Gorter's data. Maximum spreading took place at pH 1 and at the isoelectric point. Hemocyanin and vaccinia elementary bodies showed no spreading at their isoelectric points, but below pH 3.3 both exhibited definite spreading. It may be noted that there is a correlation here with the pH stability. The vaccinia elementary bodies moved out slowly on the surface of the tray solution at any pH, there was no evidence of settling. However, with the suspension of organisms, and the tobacco mosaic virus protein, even at pH 1, an undeterminable amount failed to remain on the surface, since an easily visible ring could be seen settling to the bottom of the tray when either of these materials was applied to the surface. For this reason, it is possible that the failure to demonstrate surface films depends on the technical difficulty of bringing these proteins onto the surface without loss by settling.

In an attempt to eliminate this source of error, tray solutions of greater density, 25 to 90 per cent saturated ammonium sulfate, were used.<sup>1</sup> Preliminary observations using the loop of paraffined thread described by Langmuir showed that bacterial suspensions would

<sup>1</sup> Reagent grade adjusted to about pH 7 with ammonia.

spread out with almost explosive rapidity on the surface of such solutions and displace the floating thread. However, after a few seconds the thread would of itself return to its former position. The same occurred with tobacco mosaic virus protein. When it was found that distilled water would bring about the same phenomenon it was realized that although the suspended material remained on the surface, the apparent spreading was due to a transitory water effect.

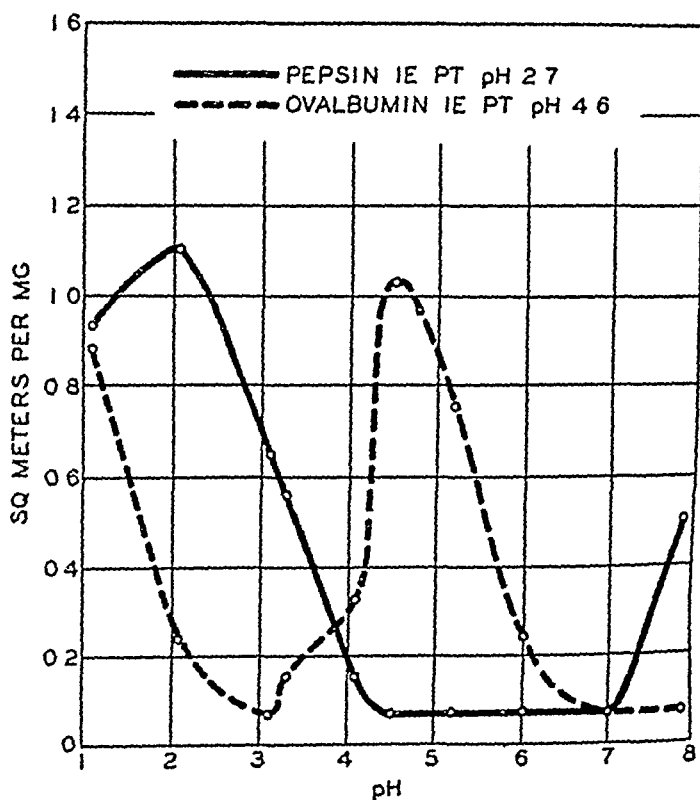


FIG 1

This is borne out in the data presented in Fig 3. Under conditions which cause pepsin and ovalbumin to spread to 1 sq meter per mg, the area occupied by the bacteria, and by tobacco mosaic virus, is very small. Hemocyanin occupies about one-fourth the area of the lower molecular weight proteins, while the elementary bodies of vaccinia spread to about one-seventh of 1 sq meter per mg. There can be no doubt that the materials in question were on the surface

when these measurements were made. In addition to the gross observation of the bacterial suspension moving out from the loop, plate counts of the solution under the surface showed that about 99 per cent of the bacteria had remained on the surface. Compression of the surface area caused the organisms to gather into a visible sheet which could be removed on slides and examined microscopically. It consisted of closely packed organisms which had apparently under

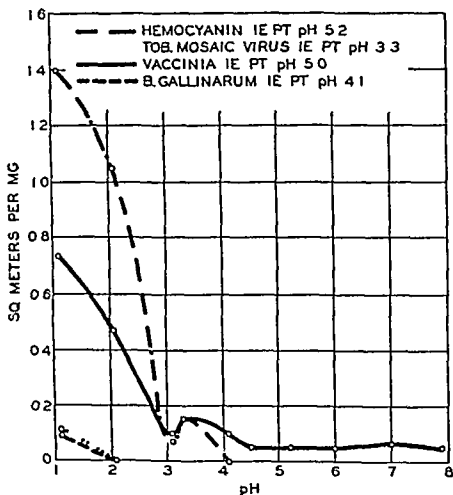


FIG 2

gone two-dimensional agglutination. Such aggregation of non-spreading particles would constitute a serious source of error in any activity measurements on surface films.

The tobacco mosaic virus protein was also shown to remain on the surface of ammonium sulfate solutions. If the extremely small films developed by this material resulted from the fact that 99 per cent of the protein entered the subsurface or substrate solution, one should

be able to demonstrate the virus there. Accordingly, virus activity measurements<sup>2</sup> on the liquid under the film were carried out.

0.2 cc containing 1 mg of virus protein was allowed to flow slowly onto the surface of the tray containing half-saturated ammonium sulfate solution. After measuring the surface area, a 10 cc pipette was introduced into the tray behind the barrier in as nearly horizontal

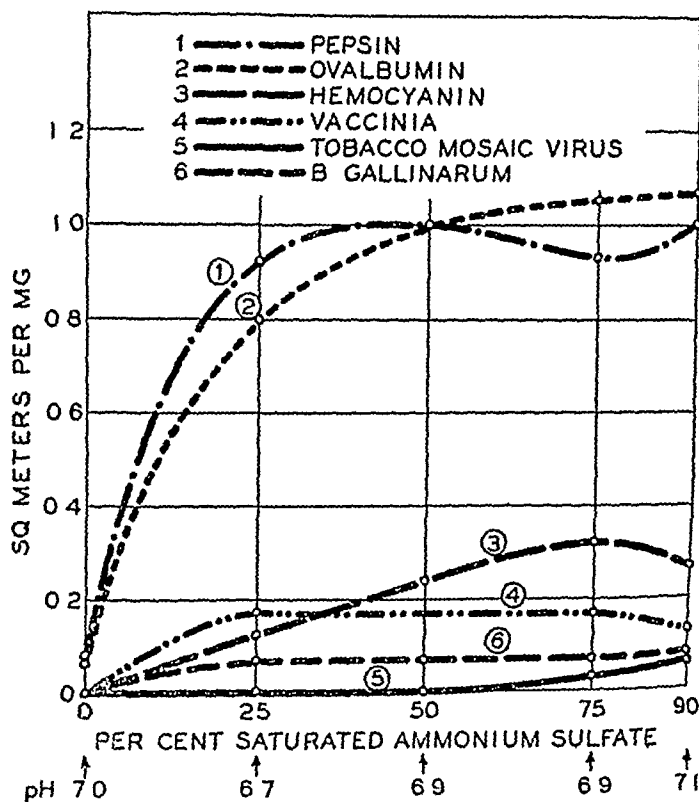


FIG 3

a position as possible. The pipette was filled and vigorously discharged 15 times causing a definite swirling of the tray contents. 10 cc were withdrawn, dialyzed 3-4 hours in a rocking dialyzer at about 6°C and inoculated onto leaves. The final ammonium sulfate concentration was less than 0.5 per cent. Controls consisted of the same

<sup>2</sup> The author wishes to express his gratitude to Dr. H. S. Loring for assistance in carrying out these activity measurements.

amount of virus discharged *under* the surface of fresh tray solutions, stirred, dialyzed, and inoculated in exactly the same manner

In the titration of a substrate solution under a film of 0.016 sq meter per mg, only 1 lesion appeared on 32 inoculated leaves, in the control, with all conditions identical except that the virus protein was introduced beneath the surface, there were 101 lesions on 23 inoculated leaves. In another similar experiment using the same virus preparation, 1 mg gave a film of 0.012 sq meter. The sample re

TABLE I

	Diameter of particle	Observed area covered by 1 mg spread on 90 per cent saturated ammonium sulfate	Calculated maximum area of 1 mg as spherical particles
	cm	sq meters	sq meters
Pepsin	$5.5 \times 10^{-7}$ (17)	1.00	0.24
Ovalbumin	$4.3 \times 10^{-7}$ (13)	1.07	0.26
Hemocyanin	$9.0 \times 10^{-7}$ (13)	0.27	0.14
Tobacco mosaic virus	$1.5 \times 10^{-6}$ (16)	0.06	0.09
Vaccinia	$1.5 \times 10^{-5}$ (18)	0.13	0.009
<i>B. gallinarum</i>	$>1.0 \times 10^{-4}$	0.07	

\* These values were obtained by multiplying the number of particles in 1 mg by the area of a square the sides of which equalled the diameter of the particle in question. The calculated area represents a maximum value, since circles actually pack into an area *less* than that occupied by the same number of squares whose sides equal the diameter. The number of particles was obtained by dividing the volume of 1 mg (assuming the specific gravity to be 1.4) by the volume of a single particle determined from its diameter. Surface area in sq meters

$$0.67 \times 10^{-7}$$

= molecular radius in cm

moved from underneath this film and inoculated on 18 leaves showed no lesions, the control showed 27 lesions on 18 leaves. In a third determination the substrate solution gave 1 doubtful lesion on 70 leaves, the control, in which the virus was introduced beneath the surface, showed 78 lesions on 70 leaves. While one would hesitate to conclude that 99 per cent of the virus protein remained on the surface, it seems clear that there is very little loss into the substrate.

In Table I, the observed areas in square meters per milligram on 90

per cent saturated ammonium sulfate solution are presented and compared with the theoretical areas which a monolayer of contiguous spherical molecules would occupy. Pepsin and ovalbumin occupy about 4 times the theoretical area. Hemocyanin occupies twice the expected area, in calculating this area a certain amount of error is introduced since *Limulus* hemocyanin molecules are not spherical (13). The film of tobacco mosaic virus is smaller than that calculated on the basis of packed spheres. The molecule of this protein is also rod-shaped (14) and may be capable of packing into an area smaller than that calculated for a spherical molecule. Vaccinia and the organism suspension spread out to form a film which, though small, was much larger than the calculated area.

The presence of any low molecular weight protein impurity or breakdown product of a larger particle should be considered as a source of error, especially in the interpretation of a film as small as that produced by *B. gallinarum*. For example, it was found that a bacterial suspension after standing in the ice box for 10 days gave a surface film of 0.4 sq meter per mg. However, after sedimenting the bacterial bodies, the almost clear supernatant spread to the same extent.

In spreading at pH 1 it is probable that two factors are operating. One is the effective electrolyte in 0.1 N HCl which would bring about spreading regardless of the H ions. The other is the effect of the H ions in breaking down certain proteins into smaller components. Both *Limulus* hemocyanin (9) and vaccinia (15) are unstable below pH 3 to 4, which accounts for their spreading below this pH level.

From the measurements presented it may be concluded that not all proteins spread in the same way as the low molecular weight proteins such as ovalbumin and pepsin. This is in agreement with data on respiratory proteins obtained by Philippi (7). A relationship between molecular weight and spreading capacity is suggested. The rapid spreading of low molecular weight proteins at their isoelectric points could not be duplicated with hemocyanin, tobacco mosaic virus, vaccinia, or a suspension of bacteria.

The calculated thickness (volume of protein divided by film area) of films of tobacco mosaic virus protein on half saturated solutions of ammonium sulfate is about 1000 Å. If the molecules are horizontally oriented, it is obvious that such thick films cannot be monomolecular. It is possible that they may be monomolecular if a vertical molecular

orientation occurs, similar to that of a stearic acid monolayer (8) X ray data indicate a molecular diameter of about 150 Å (14) If the film thickness (corresponding to the molecular length) were 1200 Å, 1 mg of vertically oriented molecules in a surface film would occupy 76.5 sq cm This value is of the same order of magnitude as the observed area of 1 mg, and molecules of these dimensions, having a density of 1.37 would have a molecular weight of about 17 million Although the existing data on the molecular dimensions and molecular weight of the tobacco mosaic virus protein would permit the assumption of a monolayer of vertically oriented rod shaped molecules, the rather tentative nature of these data must be borne in mind

The author wishes to express his gratitude to Dr J H Northrop for his invaluable advice and suggestions

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# EFFECTS OF POTASSIUM ON THE POTENTIAL OF HALICYSTIS

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(Accepted for publication, January 6, 1938)

Potassium alters the  $P.D.$  of *Halicystis*<sup>1</sup> and of *Valonia*<sup>2</sup> in the negative<sup>3</sup> direction. The experiments appear to indicate that  $U_K$ , the apparent mobility of  $K^+$  in the protoplasmic surface, is greater than that of  $Na^+(U_{Na})$  and that of  $Cl^-(V_{Cl})$ .

To study the effects of potassium on *Halicystis* artificial sea water was prepared in which the sodium was replaced by potassium; this will be referred to as "K sea water"<sup>4</sup>. This was applied to the cell<sup>5</sup> with and without dilution<sup>6</sup>.

The effects of potassium are illustrated in Fig. 1, which shows the result of raising the concentration of potassium in the sea water from 0.012 M to 0.52 M ("K sea water").

Fig. 2 shows the effect of various dilutions of "K sea water". This is of special interest in view of Damon's findings with *Valonia*. In sea water, *Valonia* usually has a negative  $P.D.$  of 5 to 10 mv. When sea water is replaced by K sea water the  $P.D.$  becomes about 60 mv more negative. Dilution<sup>7</sup> of K sea water lessens the negativity but

<sup>1</sup> Blinks, L. R. *J. Gen. Physiol.* 1932-33, 16, 147.

Damon, E. B. *J. Gen. Physiol.* 1932-33, 16, 375.

<sup>3</sup> The  $P.D.$  is negative when the positive current tends to flow from the external solution across the protoplasm to the sap.

<sup>4</sup> This contained 0.52 M KCl and 0.012 M NaCl and the other constituents of sea water at their usual concentrations.

<sup>5</sup> The experiments were performed on *Halicystis Osterhoutii* (Blinks, L. R. and Blinks, A. H. *Bull. Torrey Bot. Club* 1930-31, 57, 389) using the technique described in a former paper (Osterhout, W. J. V. *J. Gen. Physiol.* 1936-37, 20, 13). Temperature 20-25°C.

<sup>6</sup> The dilution was made by adding a solution of 1.1 M mannite + 0.02 M  $CaCl_2$ . Cf. Osterhout, W. J. V. *Proc. Nat. Acad. Sci.* 1938, 24, 75.

<sup>7</sup> Damon used an isotonic glycerol solution to dilute the sea water. Damon, E. B. *J. Gen. Physiol.* 1937-38, 21, 383.

when the dilution reaches a certain point a striking change occurs and further dilution results in an increase in negativity.

Damon explains this (on the assumption that the P.D. is due to diffusion potential) by saying that as dilution progresses the tendency of  $K^+$  to enter becomes less and less until a critical dilution is reached at which  $K^+$  no longer tends to enter. At this point (and in higher dilutions)  $KCl$  has a negligible effect and  $NaCl$  determines the

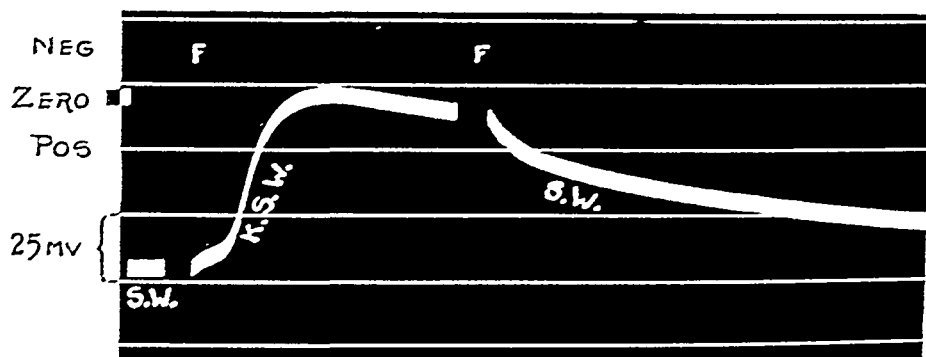


FIG. 1. Photographic record showing changes of P.D. produced by potassium (all solutions at pH 8.2). The cell in sea water at the start had a positive P.D. of 65 mv. When it was lifted out of the sea water the curve jumped to F, the free grid potential of the amplifier. It was then placed in sea water containing 0.52 M  $KCl + 0.012$  M  $NaCl$  (the other constituents of the sea water had the normal concentration) this is called "K-sea water" and is labelled K.S.W. in the record. The curve then jumped back to its former level and after a short latent period rose to 1 mv. negative and then began to drop. When the cell was replaced in sea water the P.D. became normal.

If the cells were left in K.S.W. the P.D. gradually drifted back to normal indicating a penetration of potassium. With some cells there was no latent period.

Time marks 5 seconds apart. Temperature 25°C.

A similar result was obtained with both solutions at pH 6.4.

P.D. The smaller the value of  $U_{\infty}$  (that of  $V_{Cl}$  being fixed) the smaller the critical dilution. Hence we might expect that in *Halicystis* the critical dilution would be smaller than in *Valonia* because the value of  $U_{\infty}$  appears to be less in *Halicystis* than in *Valonia*.

This is not the case. The critical dilution in *Valonia* is between 8 and 10 but in *Halicystis* a dilution of 1 — 16 has been reached without attaining a critical dilution (Fig. 2).

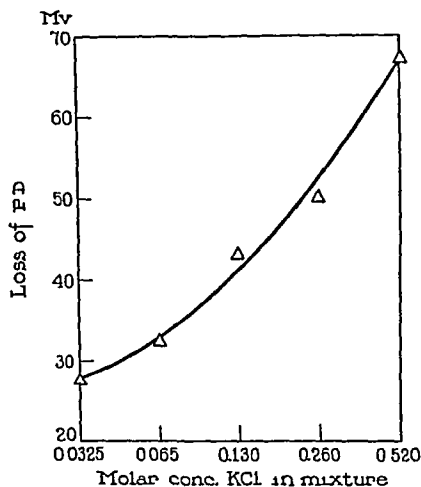


FIG 2 Curve showing effects of various dilutions of "K sea water" (cf Fig 1 and Table I)

The undiluted K sea water contains 0.52 M KCl. The scale of concentrations is logarithmic, the lowest concentration of KCl (0.0325 M) represents a dilution of 1 to 16 of K sea water.

The curve is drawn free hand to give an approximate fit.  
Temperature 20-25°C

TABLE I

*Loss of p.d. Observed on Transferring the Cell from Sea Water to "K Sea Water" or to Various Dilutions of This*

Dilution	Conc. $K^+$ in solution M	Loss of p.d. mv	No. of observations
1 - 1	0.5200	$67.5 \pm 1.3$	6
1 + 2	0.2600	$49.7 \pm 1.2$	12
1 + 4	0.1300	$43.1 \pm 1.5$	13
1 + 8	0.0650	$32.5 \pm 1.1$	17
1 + 16	0.0325	$27.8 \pm 0.7$	18

It should be said, however, that the determination of  $U_{Na}$  in *Halicystis* is uncertain and that conditions are quite different in the two organisms. In *Valonia* there is normally an inwardly directed potential of about 10 mv which tends to oppose the entrance of cations. But in *Halicystis* there is an outwardly directed potential of about 67 mv which tends to make cations enter.<sup>9</sup> The potentials therefore favor the entrance of  $K^+$  to a much greater extent in *Halicystis* than in *Valonia*. These and other differences<sup>10</sup> would seem to make a comparison between *Valonia* and *Halicystis* of rather doubtful significance.

#### SUMMARY

Sea water in which sodium has been replaced by potassium produces about the same degree of negativity in *Halicystis* and in *Valonia*.

With increasing dilution of this sea water up to 1 — 16 the degree of negativity steadily falls off in *Halicystis*. This differs from the situation in *Valonia* where Damon finds that with increasing dilution the negativity passes through a minimum after which increasing dilution produces increasing negativity. But conditions in the two organisms differ so greatly that a comparison is of rather doubtful significance.

<sup>9</sup> Teorell, T, *Proc Nat Acad Sc*, 1935, 21, 152, *J Gen Physiol*, 1937-38, 21, 107

<sup>10</sup> For differences in the composition of the sap see Osterhout, W J V, *Ergebn Physiol*, 1933, 35, 967

# BRIGHTNESS DISCRIMINATION AS A FUNCTION OF THE DURATION OF THE INCREMENT IN INTENSITY

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Recent discussions of intensity discrimination in vision have focussed attention on initial events in the process. Hecht (1935), in particular, has proposed a theory which states that brightness discrimination is due to the photochemical processes which take place at the initial moment when the eye, already adapted to a given intensity, is exposed to a just discriminably higher intensity. Results of recent observations by Smith (1936) and Steinhardt (1936) lend support to the hypothesis.

An important question which arises when we consider a theory in terms of initial events is the problem of how such a formulation may be related to the Bunsen Roscoe law. This law states that, for brief flashes of light, the product of intensity and duration is constant for the production of a constant photochemical effect. It has been found to apply within well marked limits of exposure to both the fovea and periphery of the human eye, for threshold (Graham and Margaria, 1935, Karn, 1936) and supraliminal excitation (McDougall, 1904, Blondel and Rey, 1911, Graham and Cook, 1937). Adrian and Matthews (1927) and Hartline (1928) have demonstrated the law for the eyes of lower organisms, and Hartline's findings for the single fiber of *Limulus* (1934) give adequate evidence on the nature and limitations of its application. The strict reciprocity relation fails for exposures longer than a "critical duration" beyond which, in the *Limulus* eye and probably in the human eye (Karn, 1936), the relation  $I \cdot t = \text{Constant}$  is superseded by the relation  $I = \text{Constant}$ .

These considerations have led us to perform the experiments reported here. It has seemed important to us, because of the emphasis on initial events in brightness discrimination, to determine the effect

of varying the duration of  $\Delta I$ , the just discriminable increment of intensity. We have been particularly interested in results obtained for short flashes, where initial events [e.g., a short burst of nerve impulses (Hartline, 1934)] might be expected to occur in relatively uncomplicated form and under conditions which will allow for an examination of a possible reciprocity effect of time and intensity. Data are presented on intensity discrimination for seven durations of  $\Delta I$ , and the results are related theoretically to the Bunsen-Roscoe law and to Hecht's theory.

### *Apparatus and Procedure*

The apparatus which was used in these experiments is a modification of one described by Smith (1936). The subject is seated in a cubicle which consists, in effect, of a small room within the larger, enclosing dark room. Only the back of the cubicle is open. During the experiment the subject noticed no appreciable amount of reflected light through the rear of the cubicle, and we are convinced that the shielding of the optical system precluded any possibility of anomalous results from stray light.

The optical system is constructed so as to provide two separate beams of light from the same source and equipped to permit gross variations in the intensity of both beams and fine variations in the intensity of one beam. Light from a 1000 watt lamp, after passing through a quartz cooling cell, a convex lens, and a holder for Wratten neutral tint filters, is divided by a system of four totally reflecting prisms into two beams. The two beams are centered by two pairs of matched convex lenses on semicircular apertures in a metal screen, this screen, in turn, being fastened to the front wall of the subject's cubicle. In one of the beams are a photographic wedge and balancing wedge. Manipulation of the wedge allows for an equation of the intensities on the two semicircles. The semicircles are covered with opal glass on the side toward the light source and are separated by a metal fin which projects perpendicularly from the screen in order to restrict the illumination of each semicircle to its single beam. The metal screen is attached to the front wall of the subject's cubicle in such a way that the two stimulus objects are directly in front of the subject and at the level of his eyes. Under these conditions they appear as two separate illuminated semicircles in a dark field. Viewed at a distance of 60 cm. each semicircle has a radius of 38 minutes, the separation between semicircles being 8 minutes. Thus, the total configuration subtends a visual angle of 84 minutes, and falls within the limits of the fovea. A stereoscope hood, from which the prisms have been removed, is used as a head-rest by the subject.

The apparatus, as described, makes it possible to equate (within approximately the limits of accuracy described by Smith) the intensities on the two semicircles. For the presentation of  $\Delta I$  in the form of a flash we employed a third beam of light

A mirror placed at one side of the light source reflects rays which pass through a holder for Wratten neutral tint filters a condensing lens, and a Wratten neutral tint wedge with balancing wedge. The rays finally diverge from a focus to illuminate the opal glass patch situated on the subject's left. In the focal point of the third beam is placed a device for regulating the exposure of  $\Delta I$ . For long durations (0.03 second to 0.5 second) we used a synchronous motor driven exposure device similar to one described by Graham and Granit (1931). This consists of two semicircular cardboard disks which may be caused to overlap by various degrees thus giving different widths of exposure opening. The disks are attached by a shaft through reduction gears to a telechron synchronous motor. Pressure by the experimenter on a button releases a pin which holds the shaft in place and closes the switch which starts the motor. The shaft is stopped automatically at the end of one revolution by the pin and by the breaking of a mercury switch which is operated by a cam. The shaft turns at the rate of 1 revolution per second.

For durations of 0.03 second and shorter, we used a device which consists of a synchronous phonograph motor to the axle of which is attached a large cardboard disk. A variable slit cut near the periphery of the disk allows for changes in the duration of exposure of the third beam. Since the motor is kept running all the time, the light, as it passes through the slit in the disk, flashes at the rate of once every 0.77 second. In order to restrict the illumination to single flashes, a hand-operated shutter is placed in the third beam between the opal glass and the disk of the exposure device. With practice it soon becomes possible for the experimenter to open the shutter at an interval before the slit of the disk passes through the focus of the third beam and to close it an interval after the emergence of the slit. In order to facilitate this procedure a small triangle of white paper is placed on the periphery of the disk at about a distance of 90° from the slit opening. This object can be seen rotating in the dim illumination provided by the apparatus and serves to mechanize the experimenter's timing of the hand-operated shutter. Thus, only when the hand-operated shutter is released is a flash let through to the milk glass surface and the duration of this flash is determined by the size of the slit in the cardboard disk of the phonograph motor.

We feel that the error due to lack of 'suddenness of onset' of the flash is small except for the shortest duration (0.002 second). The focus of the third beam consisted in these experiments of a small spot of light of about 1 mm. width, but a slight haze about the spot caused the total image to have a width of about 2 mm. Since the slit for the shortest exposure was 5 mm. in width, it is obvious that the waveform of distribution of light in time was by no means rectangular for this particular duration. For other durations, however the error is slight.

The procedure on any day consisted in varying the photographic wedge in the second beam until the subject reported both beams as equal in intensity. This procedure was usually accomplished at a fairly high intensity of the two patches and always with the third beam occluded. Day to day variations in the equation point were relatively small and of about the same order of magnitude as those

reported by Smith. After the equation point had been determined, determinations were made to find the necessary increment,  $\Delta I$ . The wedge in the third beam was placed in such a position that a clearly visible flash of light was superimposed upon the left hand semicircle, and then the experimenter determined the threshold by decreasing the intensity of this beam. Single flashes of  $\Delta I$  were allowed to stimulate the eye of the subject at approximately 10 second intervals. Since the subject was constantly adapted to the prevailing equated intensity on the two patches and since the intensity of the third beam was relatively small in comparison with the prevailing intensity, it may be accepted that a fairly constant level of adaptation was maintained at any intensity. Once the threshold for the given duration of flash had been determined for a given prevailing intensity,  $I$ , the filters in the third and in the divided beam were changed and determinations for  $\Delta I$  were instituted at another intensity. This procedure was followed on any day for a given exposure time for eleven prevailing intensities (ten for the 0.002 second duration). In half of the series, determinations progressed from the lowest intensity level to the highest, and in the other half determinations were started at the highest intensity and progressed to the lowest. Because of the continued adaptation at any given intensity, the results for both series show a high degree of agreement. C. H. G. served as the subject throughout the determinations and E. H. K. was experimenter. Binocular observation was used in all the experiments.

## RESULTS

The results of the experiment are presented in Table I and Fig. 1. In Fig. 1  $\log \Delta I/I$  has been plotted against  $\log I$ . Each value in Table I is the average of eight readings, except for the 0.03 second duration. The averages for this duration are based on fourteen readings of which eight were determined with the device used for shorter exposures and six with the device used for the longer exposures. Since a considerable change in apparatus took place when the exposure systems were changed, it was considered advisable to make determinations at the same duration by both devices. So far as we can see, the results obtained under the two conditions are quite similar. Two determinations were obtained in any single day's run at a particular duration of exposure.

The graph, as given in Fig. 1, indicates the general nature of the results obtained. Brightness discrimination at any given duration is represented by a high value of  $\log \Delta I/I$  at the lowest value of  $\log I$ . From the highest value of  $\log \Delta I/I$  the curve drops in a continuous manner as intensity increases, until eventually at medium to high



TABLE I  
 $\Delta I/I$  As a Function of Intensity and Duration

Log $I$ (milli lamberts)	Log $\Delta I/I$						
	0.002 sec.	0.005 sec.	0.013 sec.	0.030 sec.	0.080 sec.	0.20 sec.	0.50 sec.
2.27		-0.65	-0.98	-1.33	-1.44	-1.30	-1.35
1.67	-0.22	-0.65	-0.99	-1.27	-1.34	-1.20	-1.40
1.27	-0.22	-0.60	-0.90	-1.20	-1.40	-1.22	-1.33
0.67	-0.23	-0.53	-0.93	-1.23	-1.37	-1.33	-1.30
0.27	-0.13	-0.53	-0.85	-1.09	-1.38	-1.37	-1.34
-0.33	0.08	-0.33	-0.69	-0.94	-1.26	-1.27	-1.25
-0.73	0.37	-0.09	-0.62	-0.81	-1.19	-1.23	-1.11
-1.33	0.65	0.21	-0.23	-0.57	-0.93	-0.99	-0.92
-1.73	0.89	0.45	0.01	-0.36	-0.92	-0.92	-0.83
-2.33	1.35	0.89	0.53	0.16	-0.26	-0.40	-0.34
-2.73	1.68	1.23	0.87	0.51	0.01	-0.08	-0.07

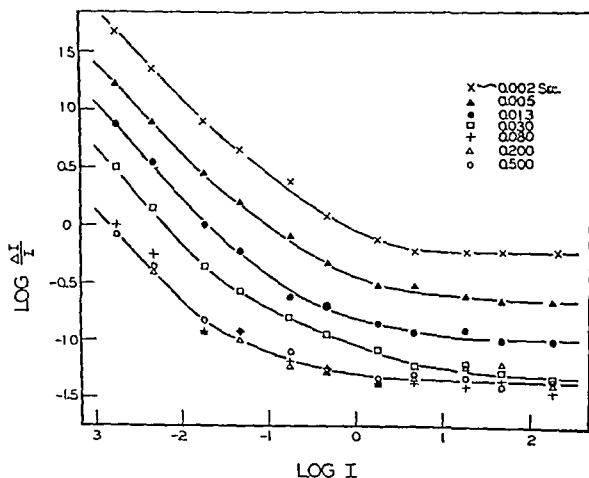


FIG. 1 The relation between  $\Delta I/I$  and  $I$  for the different durations of  $\Delta I$  used in these experiments

intensities the logarithm of  $\Delta I/I$  reaches a final steady minimum. The curve at any constant duration is similar in form to those that have been shown by Hecht (1935), Steinhardt (1936), and Smith (1936). Since our observations were restricted to a foveal region, there is no evidence of any rod portion of the curves. They are simple and continuous and exhibit no such breaks as have been shown to occur by Hecht and Steinhardt with larger fields and at lower values of intensity than we have used.

From the point of view of our interest, the important thing to note about the graphs is the position that they occupy upon the ordinate axis. The curve for the 0.002 second duration lies highest on the ordinate and the curves for the 0.005, 0.013, and 0.03 second durations are situated lower and lower in a progressive manner. At a duration of 0.08 second and beyond, the progressive downward displacement no longer takes place, and we find that the curves for 0.08, 0.2, and 0.5 second appear to be superimposed at the bottom of the graph. The curves for the three shortest exposure times, 0.002, 0.005, and 0.013 second, are parallel to one another, and the curve for 0.03 second may, within the limits of experimental error, be considered parallel to the other three. However, the superimposed curves for 0.08, 0.2, and 0.5 second cannot be considered, with all due allowance for error, to be parallel to the curves for the shorter durations.

An important characteristic to be noted in the curves for the shortest durations (with the possible inclusion of the 0.03 second curve) concerns the manner in which values of  $\Delta I$  at common abscissa values of  $I$  vary with the duration of exposure. (Since the curves have common abscissa values of  $I$  we may just as readily note the variations in  $\Delta I/I$ .) When we regard Fig. 1 with this in mind we observe something of immediate significance: the increment in intensity,  $\Delta I$ , required for brightness discrimination increases as the duration of exposure decreases. This generalization is demonstrated by the fact that the curve for the lowest duration (0.002 second) has the highest values of  $\Delta I/I$  and the curves for successively higher durations (up to 0.03 second) have successively lower values of  $\Delta I/I$ . In general, it seems that an inverse relation exists between  $\Delta I$  and exposure time, the exact form of which we shall discuss in a later section.

The progressive displacement in the values of  $\Delta I/I$  does not occur for the three curves of longest duration, and so the ordinate values are

independent of duration as a variable. In summary of these facts we may say that, within the limits of duration used, exposure time may influence the value of  $\Delta I$  at a given intensity,  $I$ , for values of duration which include 0.03 second. For durations equal to and greater than 0.08 second, exposure time has no influence on the determination of  $\Delta I$ , and the ratio  $\Delta I/I$  at a given  $I$  is constant.

### *The Bunsen Roscoe Law*

The conditions of this experiment are such that after continued adaptation to a given intensity,  $I$ , the subject is required to discriminate a brief increase in the intensity of one of the semicircles. If we consider that the determinant of this discrimination is a given increase in the amount of photolysis above the level maintained by  $I$ , then for this new photolysis the Bunsen Roscoe law should be valid and we should obtain the relation

$$\Delta I \tau = C = f(I) \quad (1)$$

where  $\tau$  is the duration of the flash, and  $C$  a constant for a given value of  $I$ . With a change in  $I$ ,  $C$  becomes a variable, since its magnitude depends upon the amount of photosensitive material present at the particular adaptation condition set up by  $I$ .

In studies such as this it is customary to test for this relationship by plotting the energy of the flash (intensity  $\times$  duration) against duration. When this procedure is followed for the data of this experiment we obtain the family of curves presented in Fig. 2. In this figure we have plotted  $\log \Delta I \tau$  (for seven exposure durations at each prevailing intensity,  $I$ ) against  $\log \tau$ . This method is convenient for the reason that, with logarithmic plotting, a line having a slope of zero represents the relation  $\Delta I \tau = C$ . The number to the left of each curve in Fig. 2 is the logarithm of the intensity,  $I$ , for which the product  $\Delta I \tau$  was calculated.

The graph demonstrates that the product of  $\Delta I$  and  $\tau$  is constant over the lower range of durations for all the levels of intensity used in these experiments. At longer durations, however, the curves show a clear cut departure from the reciprocity relation. This is evidenced by the fact that the slope of each curve changes abruptly, beyond the horizontal region, to a slope which has been drawn with a value of unity. The resulting line in each case has an equation  $\Delta I = \text{Constant}$ .

The critical duration, which sets a limit to the application of the Bunsen-Roscoe law, is the longest duration of stimulus which has an influence in determining a given aspect of the response. In the graphs of Fig 2 the critical duration is determined by the intersection of the two lines having, respectively, the equations  $\Delta I \tau = C$  (for short durations) and  $\Delta I = \text{Const}$  (for the longer durations). In Table II are entered the values of the critical durations as determined graphi-

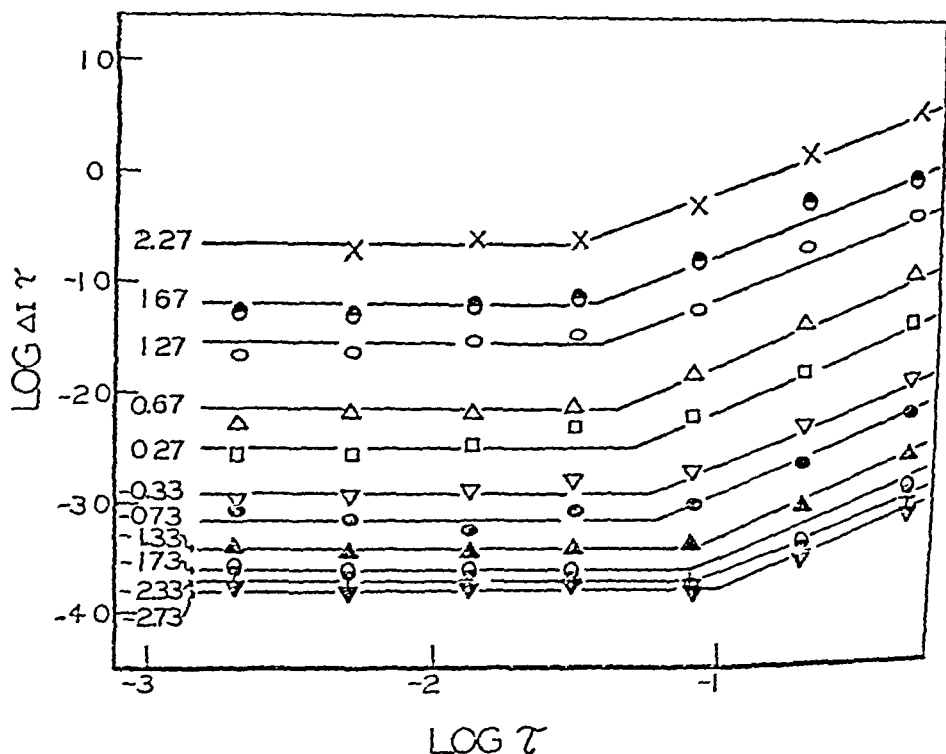


FIG 2 The relation between  $\Delta I$  and  $\tau$  for the various levels of intensity used in these experiments. The horizontal lines represent the equation  $\Delta I \tau = \text{Constant}$ , the inclined lines,  $\Delta I = \text{Constant}$ .

cally in this manner for each value of prevailing intensity. Obviously, considerable deviations from these values might still result in good fits for Fig 2, but the values are reliable enough for our purposes. Fig 2 and Table II show that the value of the critical duration is a function of intensity. In line with observations by McDougall (1904) and Graham and Cook (1937) it is found that the critical duration decreases with an increase in intensity. This variable

introduces a complicating factor into interpretations of intensity discrimination and will be discussed more fully in a later section

The existence of a critical duration and its importance in limiting the reciprocity law have been discussed by McDougall (1904), Adrian and Matthews (1927), Hartline (1934), and Graham and Margaria (1935). Hartline found in the *Limulus* eye that for durations beyond the critical duration

$$I = \text{Const} = C/\tau$$

where  $C$  is the product of  $I$  and  $\tau$  below critical duration and  $\tau_c$  is the critical duration. For the human eye this relation is not always

TABLE II  
*Critical Duration is a Function of Intensity*

Log $I$	Log critical duration
2.27	-1.50
1.67	-1.45
1.27	-1.40
0.67	-1.35
0.27	-1.30
-0.33	-1.25
-0.73	-1.20
-1.33	-1.15
-1.73	-1.10
-2.33	-1.05
-2.73	-1.00

obvious (Graham and Margaria), but it is certain that the dependence on  $\tau$  decreases with long durations. In the present experiment the result is clear and in accord with Hartline's finding. Beyond a critical duration the effect depends only on intensity, and

$$\Delta I = C/\tau = F(I) \quad (2)$$

This probably means that, as in the *Limulus* eye, where the action of the light is abruptly interrupted at the critical duration by the action of the sense cells, so in our experiment the action of  $\Delta I$  is interrupted by the increase in sensory discharge which follows the flash. As Hartline points out, this deviation from strict reciprocity cannot be

considered a failure of the Bunsen-Roscoe law in the photosensory process. When the restriction entailed by a consideration of the critical duration is recognized, we may conclude that the photochemical basis of the sensory process is a simple system to which the Bunsen-Roscoe law may be applied.

### *The Relation of the Results to Hecht's Theory*

Equations (1) and (2) indicate that the values of  $C$  and  $C/\tau_c$  are functions of intensity. This can be seen in Fig. 2 where the curve for the maximum intensity ( $\log I = 2.27$ ) is highest on the ordinate and has the greatest values of  $C$  and  $C/\tau_c$ . The curves for the other intensities are displaced downwards in a progressive manner as intensity decreases, the curve for the lowest intensity ( $\log I = -2.73$ ) lying at the bottom of the graph. A consideration of the specific function involved in this progressive displacement leads us to a theoretical interpretation of brightness discrimination. Since Hecht's theory (1935) has been successful in accounting for brightness discrimination in a number of animals, it would seem important to examine our data with the intent of providing a further test of the hypothesis.

Hecht's fundamental equation is

$$\frac{dx}{dt} = k_1 \Delta I (a - x)^m$$

which states "that the initial rate of photochemical decomposition on the introduction of the higher intensity to the photochemical system at the stationary state is proportional to  $\Delta I$  times the concentration of sensitive material at the stationary state" (1935). In this equation,  $x$  is the concentration of photoproducts broken down by the light,  $t$  is time,  $a$  is the total initial amount of photosensitive material,  $m$  is the order of the reaction, and  $k_1$  the velocity constant of the "light" reaction. Without great modification this equation may be changed to read

$$\frac{\Delta x}{\Delta t} = k_1 \Delta I (a - x)^m \quad (3)$$

which says that the increase in  $x$ , through a small but finite interval of time, is proportional to  $\Delta I$  times the concentration of sensitive material at the stationary state.

If  $\Delta t$  be accepted as equal to  $\tau$  in our experiments, equation (3) becomes

$$\Delta x = k_1 \Delta I \tau (a - x)^n \quad (4)$$

and if it be assumed that, for the discrimination of  $(I + \Delta I)$  from  $I$ , the increment in  $x$ ,  $\Delta x$ , is constant for any value of  $I$ , equation (4) becomes

$$k_1 \Delta I \tau (a - x)^n = c \quad (5)$$

where  $c$  is a constant. This equation is similar to Hecht's equation (6) except that it involves  $\tau$ , which for constant duration below critical duration may be considered as being contained in his  $k_1$ .

The steps involved in developing the relation between  $\Delta I/I$  and  $I$  are similar, from this point on, to Hecht's. For the human eye, where both forward and back reactions are bimolecular, we finally derive the expression

$$\frac{\Delta I}{I} \tau = \frac{c}{a^2 k_2} \left( 1 + \frac{1}{(kI)^{\frac{1}{2}}} \right)^2 \quad (6)$$

as a description of our experimental expectation for values of  $\tau$  below critical duration.

For values of  $\tau$  at and beyond critical duration the constant increment  $\Delta x$  must be considered as being determined within  $\tau_c$ , and equation (4) is rewritten

$$\Delta x = k_1 \Delta I \tau (a - x)^n \quad (7)$$

for the case where  $\tau$  equals or exceeds  $\tau_c$ . For these conditions equation (6) becomes

$$\frac{\Delta I}{I} \tau = \frac{c}{a^2 k_2} \left( 1 + \frac{1}{(kI)^{\frac{1}{2}}} \right) \quad (8)$$

and the value of  $\Delta I/I$  is independent of duration at a given value of  $I$ . This derivation of intensity discrimination is in accord with the implications of equations (1) and (2), as we can see if we consider the term  $(a - x)^n$  of equation (5) to be constant for a given level of  $I$ . With this assumption equation (5) becomes  $\Delta I \tau = C$ , where  $C =$

$\frac{c}{k_1(a - x)^n}$ . Similarly, if we substitute  $\tau_c$  for  $\tau$  in the same equation, the relationship is  $\Delta I = C/\tau_c$ . These are equations (1) and (2) of

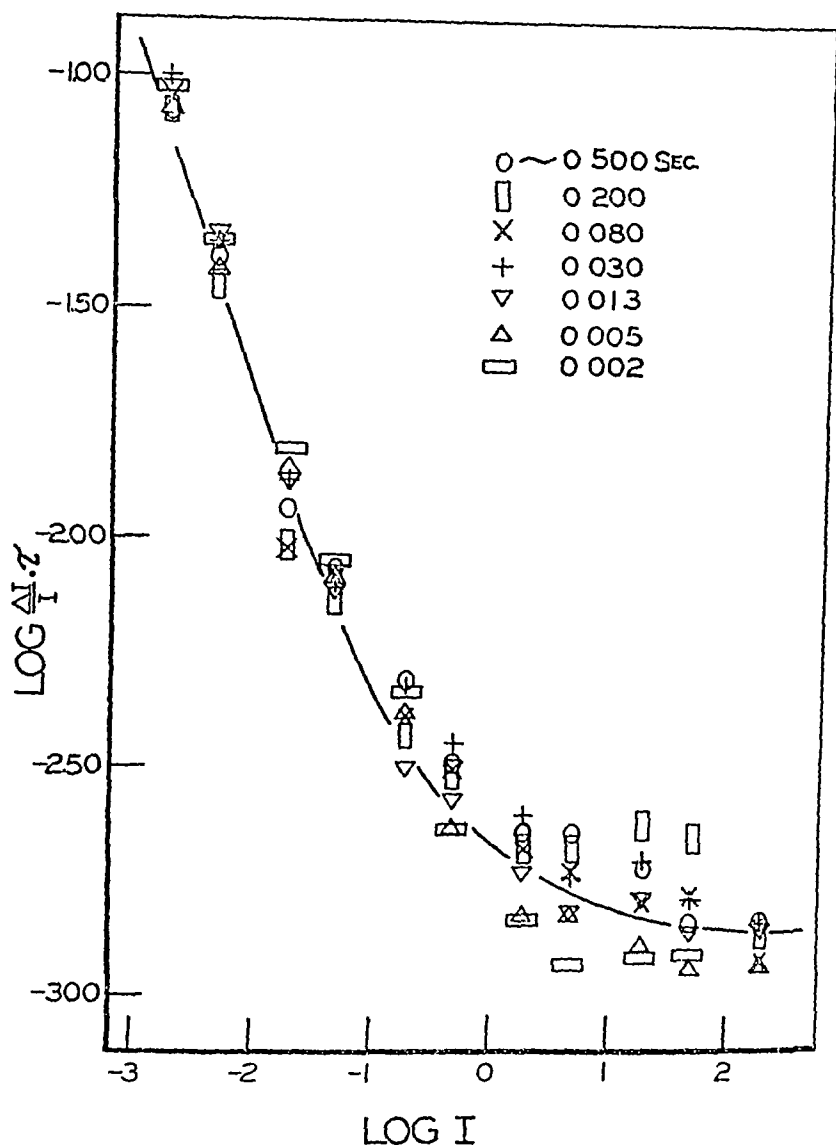


FIG 3 The relation between  $\frac{\Delta I}{I} \tau$  and  $I$  in terms of Hecht's theory. The curve is theoretical. For durations longer than the critical duration the ordinate is to be read as  $\log \frac{\Delta I}{I} \tau_c$ .

the earlier discussion, but it is significant that by these steps they are now related to Hecht's theory in a systematic manner.

The treatment of intensity discrimination given thus far would



lead us to expect that, for durations below the critical, the product of  $\Delta I/I$  and  $\tau$  should be a function of intensity, and for durations of exposure at and beyond the critical duration, the product of  $\Delta I/I$  and  $\tau_c$  should be the same function. This is true because the right hand expressions of (6) and (8) are identical for the same values of  $I$ . Practically, this means that if we plot, on a graph with  $\log I$  as abscissa, values of  $\log \Delta I/I \tau$  for all durations *below critical duration* and values of  $\log \Delta I/I \tau_c$  for all durations *at and above critical duration*, there should result a family of superimposed curves.

When the data of our experiment are treated in this way we obtain the graph of Fig. 3. In constructing this graph it was necessary to know the critical value of duration for each intensity level used. The values were obtained from Table II, and in making the graph, all values of  $\Delta I/I$  for the 0.20 and 0.50 second exposures were multiplied by the appropriate values of  $\tau_c$  as obtained from Table II. Only the values of  $\Delta I/I$  for the eight highest intensities of the 0.08 second exposure could be considered as above critical duration and they, too, are multiplied by the corresponding  $\tau_c$  values. All other values of  $\Delta I/I$  are multiplied by the appropriate values of  $\tau$ .

The graph of Fig. 3 is convincing evidence that our expectation is realized. Within the experimental error the seven curves of Fig. 3 may be considered a single curve. The line drawn through the data is the curve for equation (6) as applied to the data for the 0.013 second curve. Clearly the data for all the curves fit the theoretical line as adequately as could be desired for the conditions of the experiment.

#### DISCUSSION

Our results demonstrate that when  $\Delta I$  is added to an already existing intensity,  $I$ , in the form of a flash, its intensity value must become greater as duration becomes less if a discrimination between intensities  $I$  and  $I + \Delta I$  is to be accomplished by the subject. This is true only within certain limits of duration. Within this range of duration the requirement for brightness discrimination at a constant prevailing intensity,  $I$ , is fulfilled when the product of  $\Delta I$  and time of exposure is a constant. This is the condition implied by the Bunsen-Roscoe law for the production of a constant photochemical effect, and our results show that the law holds for brightness discrimination in the human eye.

Beyond a critical duration the reciprocity relation appears to fail and the equation  $\Delta I \tau = C$  is superseded by the relation  $\Delta I = \text{Constant}$ . Hartline (1934) has given the most adequate account of factors determining the critical duration and he points out that it is meaningless to discuss the influence of duration upon events in the nervous discharge which are over before the flash is complete. Only those durations which are shorter than the time of the appearance of the event may be considered logically. This type of reasoning must apply equally well to effects in the human eye, and it sets a logical restriction to the limits of duration within which one can adequately test for the validity of the Bunsen-Roscoe law. The change at the critical duration from the reciprocity relation to the expression  $\Delta I = \text{Constant}$  does not necessarily, in the light of Hartline's discussion, mean a failure in the reciprocity law. It may mean, rather, that the action of the light is interrupted by the increase in activity of the sense cells which follows the presentation of  $\Delta I$ . As applied to brightness discrimination, this interpretation implies that the photochemical effect of  $\Delta I$  follows the Bunsen-Roscoe law rigorously. The apparent failure of the law is due to the interruption of the action of the light by the impulses which determine the discrimination.

The Bunsen-Roscoe law states conditions for the production of a constant amount of photolysis. In our experiments the validity of the law implies that brightness discrimination is determined, at any level of photolysis due to  $I$ , by a constant increment in the photoproducts which are broken down by the action of  $\Delta I$ . This interpretation has been recognized by Hecht (1935) and his theory may be considered as accounting for brightness discrimination at constant values of duration. When duration varies, however, the theory requires a minor amplification. The change in theory is necessitated by the consideration that brightness discrimination is determined by a constant amount of photolysis rather than by its initial rate. When the theory is restated in these terms it adequately accounts for the findings of this experiment. Brightness discrimination is in accord with Hecht's theory and the Bunsen-Roscoe law for durations up to the critical duration. For durations greater than the critical duration the theory is written on the assumption that the necessary increment in photoproducts,  $\Delta v$ , is accomplished within the critical duration.

When due allowance is made in the theory for the complexities introduced by the critical duration, the hypothesis is valid for all conditions of exposure time. The steps involved in this verification have been discussed earlier.

The existence of a critical duration raises a practical problem in determinations of intensity discrimination thresholds. Where the duration of  $\Delta I$  is shorter than the critical duration,  $\tau$  in equation (6) may be thought of as being contained in the constant,  $\frac{C}{a^2 k_1}$ , but where  $\Delta I$  has a duration longer than the critical duration, equation (8) applies, and  $\tau_c$  cannot be contained in a constant because it is a function of intensity, as shown in Table II. Because of this it would seem that unequivocal results on brightness discrimination can only be obtained when we use durations of  $\Delta I$  which are well below the critical exposure time for all values of intensity.

#### SUMMARY

1 This investigation has been concerned with an analysis of brightness discrimination as it is influenced by the duration of  $\Delta I$ . The durations used extend from 0.002 second to 0.5 second.

2  $\Delta I/I$  values at constant intensity are highest for the shortest duration and decrease with an increase in duration up to the limits of a critical exposure time. At durations longer than the critical duration the ratio  $\Delta I/I$  remains constant.

3 The Bunsen-Roscoe law holds for the photolysis due to  $\Delta I$ . This is shown by the fact that, within the limits of a critical duration, the product of  $\Delta I$  and exposure time is constant for any value of prevailing intensity,  $I$ .

4 At durations greater than the critical duration the Bunsen-Roscoe law is superseded by the relation  $\Delta I = \text{Constant}$ . This change of relation is considered in the light of Hartline's discussion (1934).

5 The critical duration is a function of intensity. As intensity increases the critical duration decreases.

6 Hecht's theory (1935) accounts for the data of this experiment if it be assumed that brightness discrimination is determined by a constant amount of photolysis.

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# STUDIES IN THE PHYSICAL CHEMISTRY OF AMINO ACIDS, PEPTIDES, AND RELATED SUBSTANCES\*

## XI THE SOLUBILITY OF CYSTINE IN THE PRESENCE OF IONS AND ANOTHER DIPOLAR ION

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An adequate theory of solutions for biological systems must depend upon the development of laws defining the mutual interaction, in each others presence, of ions and such dipolar ions<sup>1</sup> as amino acids, peptides, phospholipids, and proteins. The interaction between ions and dipolar ions is a function of the size, shape, and electrical moments of the latter, and also of the ionic strength and dielectric constant of the solution (5, 7). Precisely as the interaction between ions and one dipolar ion species may be shown to be diminished by the concentration of a second dipolar ion, so also increase in ionic strength may be shown to diminish the interaction between two species of dipolar ions. There is thus a multifunctional relation between the physical chemical behavior of the ionic and the dipolar ionic components of biological systems as a function of temperature, ionic strength, and dielectric constant, as well as of their specific properties.

Unquestionably, the most important dipolar ions, as their name implies, are the proteins. In order to increase our knowledge of this class of molecules we have found it desirable, however, at each stage of our investigation, first to complete comparable investigations upon simpler molecules of known structure. As a preliminary therefore to the systematic investigation of systems containing neutral salts and more than one protein, we have investigated systems containing

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<sup>1</sup> As a translation of the word "zwitter ion" we have tentatively adopted the term "dipolar ion" although it is not an ideal description of this class of molecules

TABLE I

Density $\rho$	Glycine moles $n_1$	Electrolyte moles $n_2$	Water moles $n_3$	Solubility of cystine		Log mole fraction		$D \log \frac{N}{N^*}$	$\frac{D_0}{D} \frac{I}{2}$	$\frac{D}{D_0} \log \frac{N}{N^*} + K_s \frac{I}{2}$
				Moles $n_4$	Mole fraction $N$	$\log N$	$\log N^*$			
Solubility of cystine in water $D = 78.54$										
0.9972	0	0	55.34	0.000454	0.00000820	-5.086		0	0	0
Solubility of cystine in NaCl $K_s = 0.14$										
0.99926	0	0.05	55.30	0.000478	0.00000863	-5.064		0.022	0.05	0.029
1.0014	"	0.10	55.25	0.000493	0.00000891	-5.050		0.036	0.10	0.050
1.0014	"	0.10	55.25	0.000496	0.00000896	-5.050		0.036	0.10	0.050
1.0054	"	0.20	55.15	0.000520	0.00000939	-5.027		0.059	0.20	0.087
1.0054	"	0.20	55.15	0.000520	0.00000940	-5.027		0.059	0.20	0.087
1.0176	"	0.50	54.85	0.000578	0.0000104	-4.983		0.103	0.50	0.173
1.0372	"	1.00	54.32	0.000650	0.0000118	-4.929		0.157	1.00	0.297
1.0737	"	2.00	53.10	0.000714	0.0000130	-4.886		0.200	2.00	0.480
1.1475	"	4.00	50.69	0.000845	0.0000155	-4.810		0.270	4.00	0.830
1.01279	0.5	0	54.13	0.000558	0.0000102	-4.991	-4.991	0	0	0
1.02069	"	0.20	53.91	0.000602	0.0000110	-4.959	-4.991	0.036	0.175	0.065
1.03256	"	0.50	53.60	0.000654	0.0000120	-4.921	-4.990	0.079	0.437	0.149
1.05190	"	1.00	53.05	0.000706	0.0000129	-4.889	-4.990	0.116	0.874	0.256
1.08925	"	2.00	51.88	0.000780	0.0000143	-4.845	-4.988	0.164	1.748	0.444
1.02801	1.0	0	52.89	0.000632	0.0000117	-4.932	-4.932	0	0	0
1.04740	"	0.5	52.34	0.000702	0.0000130	-4.886	-4.931	0.0580	0.389	0.128
1.04722	"	0.5	52.33	0.000696	0.0000129	-4.889	-4.931	0.0541	0.389	0.124
1.06632	"	1.0	51.77	0.000750	0.0000139	-4.857	-4.931	0.0953	0.777	0.235
1.06615	"	1.0	51.76	0.000741	0.0000138	-4.860	-4.930	0.0902	0.777	0.230
1.10313	"	2.0	50.57	0.000799	0.0000149	-4.827	-4.927	0.1288	1.554	0.409
1.05755	2.0	0	50.36	0.000743	0.0000142	-4.848	-4.848	0	0	0
1.07611	"	0.5	49.77	0.000779	0.0000149	-4.827	-4.847	0.0315	0.318	0.102
1.09454	"	1.0	49.17	0.000816	0.0000156	-4.807	-4.846	0.0615	0.635	0.202
1.13027	"	2.0	47.91	0.000833	0.0000160	-4.796	-4.843	0.0741	1.270	0.354
1.08033	2.8	0	48.29	0.000779	0.0000152	-4.818	-4.818	0	0	0
1.09817	"	0.5	47.66	0.000794	0.0000156	-4.807	-4.816	0.0163	0.277	0.086
1.11640	"	1.0	47.05	0.000800	0.0000157	-4.800	-4.815	0.0271	0.554	0.167
1.15121	"	2.0	45.73	0.000813	0.0000161	-4.793	-4.812	0.0343	1.108	0.314

TABLE I—*Concluded*

Density $\rho$	Glycine moles $n_1$	Electrolyte moles $n_2$	Water moles $n_3$	Solubility of cystine		Log mole fraction		$\frac{D}{D_0} \log \frac{N}{N_0}$	$\frac{D_0}{D} \frac{L}{L_0}$	$\frac{D}{D_0} \log \frac{L}{L_0} + \frac{L}{L_0}$
				Moles $n_4$	Mole fraction $N$	$\log N$	$\log N$			
Solubility of cystine in $\text{Na}_2\text{SO}_4$ $\lambda = 0.18$										
1 0091	0	0 10	55 22	0 000533	0 00000963	-5 016		0 070	0 30	0 124
1 0280		0 25	55 08	0 000579	0 0000105	-4 979		0 107	0 75	0 242
1 0587		0 50	54 81	0 000616	0 0000111	-4 955		0 131	1 50	0 401
1 1157		1 00	54 03	0 000645	0 0000117	-4 932		0 154	3 00	0 694
1 1273		1 10	53 89	0 000649	0 0000118	-4 928		0 158	3 30	0 752
1 1441		1 25	53 64	0 000621	0 0000113	-4 947		0 139	3 75	0 814
1 1632		1 41	53 41	0 000612	0 0000112	-4 951		0 135	4 23	0 896
1 1652		1 50	52 84	0 000595	0 0000109	-4 963		0 123	4 50	0 933
1 04317	0 5	0 25	53 84	0 000656	0 0000120	-4 921	-4 991	0 080	0 656	0 211
1 07257		0 50	53 50	0 000697	0 0000128	-4 893	-4 990	0 110	1 311	0 374
1 08627	2 0	0 25	49 98	0 000760	0 0000146	-4 836	-4 848	0 019	0 476	0 150
1 81387		0 50	49 54	0 000755	0 0000145	-4 839	-4 846	0 011	0 953	0 274

neutral salts and two amino acids. Adopting the method of solubility we have chosen cystine as saturating body because it is the least soluble of the amino acids, and because its S S grouping, and accurate colorimetric methods for its estimation, render it possible accurately to determine its concentration in the presence of other amino acids. Glycine was chosen as the second amino acid because the structural configuration (4) and the electrical moment (11) of this simplest dipolar ion are now better known than that of any other.

Two salts have been investigated, sodium chloride, which increases the solubility of cystine at all concentrations that have been studied, and sodium sulfate, which increases the solubility of cystine in dilute solution, but which has a "salting-out" effect in more concentrated solution (5<sup>3</sup>, 13). Thus the influences of neutral salts on cystine have been found to be very similar to those upon proteins (5).<sup>4</sup> Studies upon this tetrapolar amino acid might therefore serve to reveal certain

<sup>2</sup> Cohn (4) Fig. 1<sup>3</sup> Cohn (5) Fig. 5<sup>4</sup> Cohn (5), Figs. 5 and 7

of the forces that obtain in systems containing electrolytes and proteins

### *Materials and Methods*

The methods for the purification of the glycine (6), of the cystine, and of the sodium chloride and sodium sulfate employed in this investigation have previously been described (13). The pure solvents were prepared by weighing glycine and sodium chloride, or sodium sulfate, into volumetric flasks which were then brought to volume with distilled water. The concentration of glycine and of salt in the solvents, given as mole per liter, are recorded in the second and third columns of the accompanying table (Table I). Because of the very low solubility of cystine the density of the solvent, given in the first column of Table I, may be considered equal to the density of the solution.

The solubility of the cystine in these solvents was always determined colorimetrically by means of the Folin uric acid reagent. Comparisons with known standards were carried out with the Koenig-Martens spectrophotometer in the manner previously described (13). The solubility of cystine is given both as mole per liter,  $n$ , and as mole fraction,  $N$ .

### EXPERIMENTAL RESULTS

The results of the measurements on systems containing cystine, glycine, and sodium chloride are graphically represented in the accompanying figures (Figs 1a and 1b), in which the solubility of the cystine is always given as mole fraction,  $N$ . The lowest curve in Fig 1a gives the interaction between cystine and glycine in the absence of salt. In the systems containing glycine solubility is always higher, and higher the greater the concentration of glycine and of salt. Whereas both glycine and sodium chloride have a solvent action upon cystine, the influence of each of these solvents is to decrease the effect of the other. Thus in the presence of 2 molal sodium chloride, 2.8 mole of glycine changed the solubility only from 0.000714, the value in the absence of glycine, to 0.000813, the value in the presence of 2.8 mole of glycine, whereas the solubility of cystine in water is 0.000454 mole per liter and in 2.8 mole of glycine 0.000779 mole per liter. The action of glycine in increasing cystine solubility in water (curve 1, Fig 1a) is thus nearly 70 per cent, whereas it is only 14 per cent in the presence of 2 mole of sodium chloride (curve 4, Fig 1a). We must therefore conclude that *the interaction between these dipolar ions is a function of the sodium chloride concentration, decreasing with in-*



*creasing ionic strength* The application of this principle to other amino acids and proteins is being further studied

The converse to the above statement regarding the influence of ionic strength on the interaction between dipolar ions may also be made For our results demonstrate that *the interaction between ions and the dipolar ion cystine is a function of the other dipolar ions in solution, diminishing with increasing glycine concentration* The very profound solvent action of sodium chloride on cystine is greatly reduced in the presence of even a mole of glycine, and is almost negligible

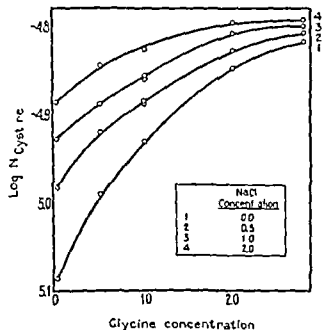


FIG 1a

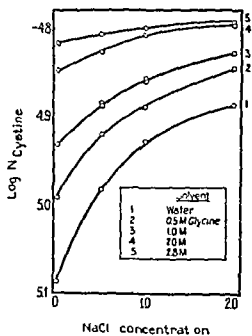


FIG 1b

FIG 1a Influence of sodium chloride on the interaction between glycine and cystine

FIG 1b Influence of glycine on the interaction between NaCl and cystine

in the presence of a 2.8 molal glycine solution In so far as the interaction between the sodium and chloride ions and the charged groups of the tetrapole cystine depends upon electrostatic forces, this effect is understandable, for by definition change in free energy due to electrostatic forces must, according to Coulomb's law, diminish inversely as the second power of the dielectric constant, and the dielectric constant of a 2.8 molal glycine solution is 141.8, or as much above water as water is above butanol In these terms an explanation may therefore be sought for the very profound differences between the curves in Fig 1

Cystine is sufficiently insoluble so that the ratio of the solubility in any of the systems studied to that in any other system, considered as standard state, yields an activity coefficient. For the purpose of analyzing further the interaction between cystine and sodium chloride, or cystine and sodium sulfate, we may define the standard state as the solubility in the system containing the same concentration of glycine, but in which the ionic strength is zero. In the case of four component systems, however, some further definition must be made in relating the properties of the different solutions. The simplest procedure would be (1) to assume that the water displaced by salt in the systems studied has no appreciable effect on either the solubility of cystine or the dielectric constant of the solution.  $N'$  is defined as the solubility in terms of mole fraction in the absence of salt according to this assumption, and is the value obtained for the systems without salt upon which such measurements were made.

A better procedure (2) might be to consider the standard state for each system containing salt as that of a system containing the same mole fraction of glycine and water, but no salt. By calculating the mole fraction of glycine,  $n_1/(n_1 + n_3)$ , in each system containing salt, it has been possible to estimate the solubility,  $N''$ , defined in this way from a curve in which measurements of  $\log N$  were plotted against  $n_1/(n_1 + n_3)$ . Values of  $\log N''$  computed in this manner are also given in Table I. The differences between  $\log N'$  and  $\log N''$  never exceed 0.003 in the systems containing 0.5 molal glycine or 0.006 in the systems containing 2.8 mole of glycine.

A further assumption must be made in order to obtain an estimate of the dielectric constant in the solutions containing neutral salts (16). Since the conductivity of such solutions precludes their being measured directly, the simplest procedure (1) would be to take the dielectric constant of the salt-free glycine-water systems employed in the solubility measurements (20) as applying also to the systems containing salt (7).

Two possible objections might be made to this procedure. On the one hand, it might be argued that the dielectric constant will not, on this basis, be equal to that in the standard state as defined by our second procedure above. The latter will be higher in glycine concentration (mole per liter) by an amount that may be estimated by the relation

$$C_{\text{glycine}} = 55.34(n_1/(n_1 + n_3)) - 78(n_1/(n_1 + n_3))^2 \quad (1)$$

On this basis (2) the concentration of glycine in the presence of 2 molal sodium chloride would be 0.521 instead of 0.5 in the salt free system that was measured, and the dielectric constant would be 90.31 instead of 89.81. The effect would be greater in the 2.8 molal glycine solution, the solubility of which in 2 molal sodium chloride on the above basis should be referred to a system containing 2.934 mole of glycine and having the dielectric constant of 144.85 instead of 141.82.

Whereas estimation of the dielectric constant of the solutions chosen by the second method for the standard state leads to a higher value of the dielectric constant, other considerations suggest lower values than those of the systems measured. Thus the displacement of a certain number of water molecules by salt molecules might be expected to slightly diminish the dielectric constant of the solution. If we assume (3) that each mole of water has the same effect in all of

TABLE II

*Estimation of the Dielectric Constant of the Standard State and of the Salt Solutions*

Glycine concentration	Calculation method	NaCl concentration $F/2$			
		0	0.5	1.0	2.0
		Dielectric constant $D = 27.6C + 78.54$			
0	(2)	78.54	78.54	78.51	78.54
	(3)		77.85	77.11	75.40
0.5	(2)	89.84	89.95	90.07	90.31
	(3)		89.10	88.33	86.69
1.0	(2)	101.14	101.38	101.61	102.13
	(3)		100.38	99.58	97.90
2.0	(2)	123.74	124.24	124.73	125.82
	(3)		122.91	122.07	120.31
2.8	(2)	141.82	142.52	143.27	144.85
	(3)		140.94	138.84	138.24

the systems studied, we may make a simple correction for this factor by subtracting the quantity  $(78.541)/55.344 = 1.4$  for each mole of water displaced. According to this calculation the influence of the salt on the dielectric constant depends upon the water displaced.

Assuming that salt does not contribute to the dielectric constant of the solution, the influence of 2 mole of salt on the 0.5 mole glycine, instead of increasing  $D$  from 89.84 to 90.31, diminishes it to 86.69 and for the 2.8 mole glycine instead of increasing  $D$  from 141.82 to 144.85 diminishes it to 138.24. These two assumptions, illustrated by the data in Table II are opposite in direction and approximately equal in magnitude. All of our results have been calculated in these ways and no essential differences in the results obtained were observed. We have therefore tentatively chosen the simplest procedure of employing the dielectric constant of the salt free systems measured (column 3, Table II) in the following calculations.

## DISCUSSION OF RESULTS

In our studies at low dielectric constant we have corrected for Coulomb forces by multiplying the logarithm of the solubility ratio by the ratio of dielectric constant of the solution,  $D$ , to that of water,  $D_0$ , and the electrolyte concentration by  $D_0/D$  (3, 5). The quantity  $(D_0/D)(\Gamma/2)$  is proportional to  $\kappa^2$  in Debye's electrostatic force theory. In the case of interaction between ions and dipolar ions terms in  $\kappa$ , or the square root of  $(D_0/D)(\Gamma/2)$ , vanish (17), and the first term is therefore in  $\kappa^2$ . If therefore we plot the measurements, made at different glycine concentrations, and therefore at different dielectric constants, with  $(D_0/D)(\Gamma/2)$  as abscissa and  $(D/D_0) \log N/N'$  as ordinate, all the curves should coincide provided the interaction between ions and dipolar ions at such high dielectric constants were completely ascribable to Coulomb forces.

Although our studies upon glycine in regions of low dielectric constant indicate that as a first approximation Coulomb forces suffice for the interaction between glycine and lithium chloride (3, 5, 11), the measurements now reported upon cystine in systems containing glycine and sodium chloride are not adequately described by this assumption. Indeed, curves plotted in the above manner do not appear even to yield the same limiting slope at low values of  $\kappa^2$ , suggesting therefore that another term must be considered.

In the case of proteins one must take into account both the solvent and the salting-out effect of neutral salts (1, 2, 9, 18, 19). The salting-out effect has also been shown to be important for gases, for such organic molecules as succinic acid (12), for electrolytes (10, 15), and for certain amino acids (8, 14). Among these cystine is especially interesting for it exhibits both the solvent and salting-out effect characteristic of salts and of proteins. For the case of cystine in systems containing sodium chloride and glycine, we tentatively defined the limiting slope in terms of the equation (5,<sup>5</sup> see also 13)

$$\frac{D}{D_0} \log \frac{N}{N'} = \left[ K_R - \frac{D}{D_0} K_s \right] \frac{D_0 \Gamma}{D} \frac{\Gamma}{2} = \left[ 0.56 - \frac{D}{D_0} 0.14 \right] \frac{D_0 \Gamma}{D} \frac{\Gamma}{2} \quad (2)$$

On the basis of this equation, where  $K_R$  and  $K_s$  are constants, the latter for the salting-out effect, were we to plot  $(D/D_0) \log N/N' + K_s(\Gamma/2)$

<sup>5</sup> Cohn (5), p. 271

against  $(D_0/D)(\Gamma/2)$  and give to  $K_s$  the value 0.14, all of the measurements upon sodium chloride would extrapolate to the same limiting slope for the systems here reported. Not only is this true, but the curves essentially coincide throughout their extent (Fig. 2). The same holds if the value for  $K_s$  in systems containing sodium sulfate is taken as 0.18. Since all of the measurements that are reported fall upon a single curve or family of curves when plotted in this way, we have a method of characterizing the activity coefficients of cystine in systems containing ions and other dipolar ions.

Any equation for the curve in Fig. 2 would yield values for the activity coefficients of the cystine in these systems. An empirical expression, on the basis of which the curve in Fig. 2 is constructed, has tentatively been adopted and has the form

$$\frac{D}{D_0} \log \frac{N}{N'} + K \frac{\Gamma}{2} = \frac{D_0 \Gamma}{D^2} \left[ \frac{K_R + A(D_0/D)(\Gamma/2)}{1 + B(D_0/D)(\Gamma/2)} \right] \quad (3)$$

This equation is an extension to systems of high but varying dielectric constant of the equation previously employed in describing aqueous systems containing cystine and neutral salts. Although there are four constants in this equation,  $K_R$  and  $K_s$  have the values (13), and appear to have the significance, previously ascribed to them. Kirkwood has suggested that the magnitude of the salting out effect could be determined entirely by the relation<sup>6</sup>

$$K = \frac{4\pi N e^2}{2303 D_0 K T} \frac{b^2}{a} \frac{D - 1}{2D + 1} \quad (4)$$

provided it depended upon the dipolar ion displacing a certain quantity of solvent and reducing the polarization of the solvent by the salt ion. In this expression  $b$  is the radius of the dipolar ions and  $a$  the sum of the radii of ions and dipolar ions. Taking  $b$  for cystine as 3.94 Å (5, 13) and  $a$  for cystine and sodium chloride as 5.15 Å, the value of  $K$  calculated by the above equation is 0.14, in excellent agreement with that experimentally determined. The agreement in the case of

<sup>6</sup> Personal communication. The equation given previously (Cohn E. J., *The chemistry of the proteins and amino acids*, in Luck J. M., *Annual review of biochemistry*, Stanford University, 1935, 4, equation 28) should be multiplied by  $D_0/D$  to yield the form given above and the influence of the dielectric constant

certain other ions and dipolar ions is less satisfactory, indicating that other forces are also merged in the salting-out effect

Kirkwood's theory for the interaction between spherical dipolar ions and electrolytes should yield  $K_R$  in terms of the above values of  $a$

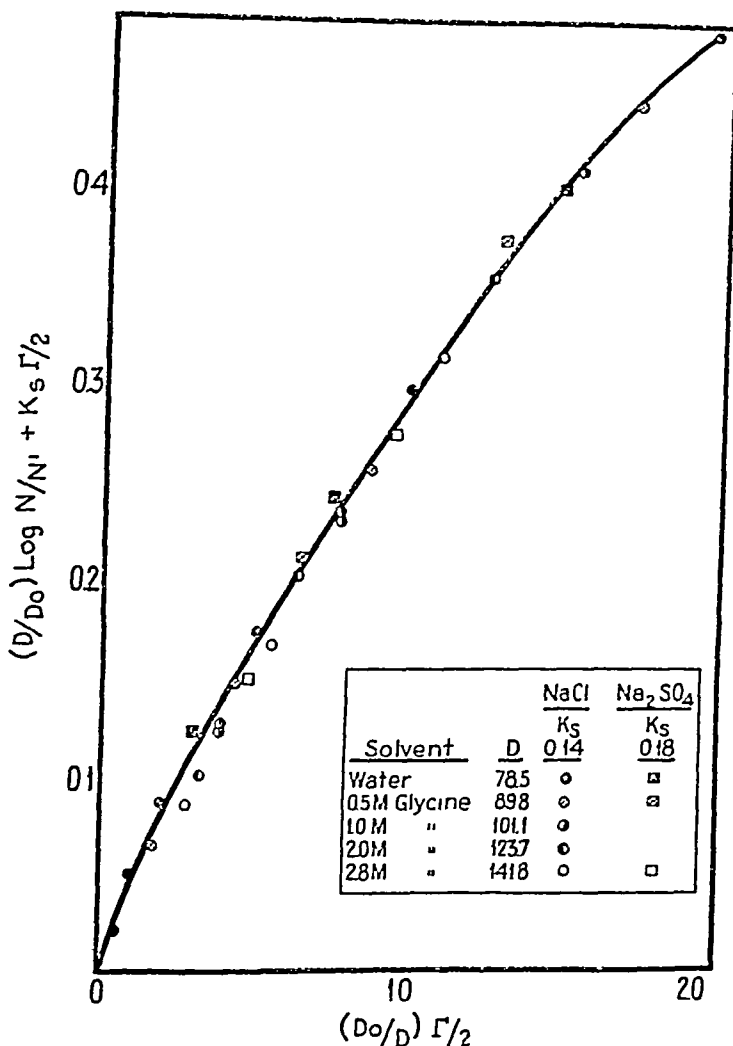


FIG 2 Interaction between glycine, cystine, and neutral salts in aqueous solutions differing in ionic strength and in dielectric constant

and  $b$  and the distance of the charged groups from each other and from the edge of the molecule. For the case of cystine, if we assume that each dipole has the same length as that of glycine, namely  $3.17 \text{ \AA}$ , and the same distance from the edge of the molecule, namely  $1.23 \text{ \AA}$ ,

the limiting slope  $K_s$  has a value close to that estimated from these experiments, provided the two dipoles are considered to be at an angle of 90 degrees to each other, the position they would assume on the basis of free rotation about the S S linkage. The solubility ratios at low ionic strength are, however, higher than those calculated with the Kirkwood equation on this assumption, but not on the assumption that the dipoles are more nearly parallel (13).

Kirkwood's equation 21 (11), expanded only through the quadrupole term, does not at higher ionic strength yield an adequate description of the shape of the curve in Fig. 2. This might not be true were all the higher terms of his equation 21 (11) taken into account, but in that case the expression would be very cumbersome. It is for this reason that equation (3) has been developed in such a form that the terms in  $K_s$  and  $K_d$  dominate at low values of the ionic strength.

For concentrated salt solutions, however, it is  $K_d$  and the terms in  $A$  and  $B$  which dominate the equation. At sufficiently high concentrations, the term  $K_s$  will become negligible and the above right hand expression will approach a straight line in respect to  $(D_0/D)(\Gamma/2)$ , having the slope  $A/B$ . For the case of cystine and sodium chloride,  $A = 0.36$  and  $B = 2.1$ . The slope  $A/B$  equals 0.17 and is greater than the value of  $K_s$ , and solubility will therefore increase in all concentrations of salt. This slope is, however, smaller than the value of  $K_d$  assumed for sodium sulfate.

The above equation is relatively insensitive to the constants  $A$  and  $B$  and even their ratio cannot be considered as well known as the constants  $K_s$  and  $K_d$ . There is very little doubt, however, that when the ratio  $A/B$  is large in comparison with  $K_s$  salt will increase solubility at all concentrations. Only when  $K_d$  is large with respect to  $A/B$  precipitation will occur, and the salting-out constants observed in very concentrated salt solutions represent the difference between the value of the above ratio and the true salting-out constant  $K_d$ , which must be taken into account at all concentrations. Thus for cystine in very concentrated ammonium sulfate (13) over the range in which the logarithm of the solubility varies inversely as the ionic strength, the apparent salting-out constant is 0.05, or approximately one-third the value 0.16 for  $K_d$  adopted from considerations such as those that have here been developed.

More significant, however, than any other relation in equation (3) is that between  $K_R$ ,  $K_s$ , and the dielectric constant. The higher the dielectric constant the smaller the value of the term containing  $K_R$  relative to that containing  $K_s$ . Thus it follows that Coulomb forces will play a smaller rôle, and the salting-out effect a larger rôle, in determining behavior the higher the dielectric constant. Since biochemical systems are generally rich in dipolar ions, and therefore will often have high dielectric constants, it follows that those forces associated with the salting-out effect must be taken into account in such systems not only in concentrated salt solutions but even in solutions of low ionic strengths.

#### SUMMARY

1 As an introduction to the relations that obtain in biochemical systems containing several components, some ionic, some dipolar ionic, the solubility of cystine has been investigated in the presence of glycine and neutral salts.

2 Both glycine and sodium chloride increase cystine solubility at all concentrations. The interaction between cystine and ions is, however, diminished with increase in glycine concentration, and the interaction between cystine and glycine with increase in ionic strength.

3 Sodium sulfate also increases the solubility of cystine, but at concentrations greater than one molal its solvent action is smaller than its salting-out effect, which is greater at all concentrations than that of sodium chloride, and greater the higher the glycine concentration.

4 These interactions are defined by an equation giving the solubility ratio of cystine in terms of salting-out constants, constants related to the electric moments of cystine, and to the ionic strength and dielectric constant of the solution.

5 The higher the concentration of glycine and therefore the dielectric constant of the solution, the smaller that part of the interaction between ions and dipolar ions which depends upon Coulomb forces and the greater appears the salting-out effect.

6 Conversely, the greater the ionic strength and the salting-out effect the smaller the interaction between dipolar ions in solution.



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# THE ACCUMULATION OF ELECTROLYTES

## IX. REPLACEMENT OF AMMONIA BY SODIUM AND POTASSIUM

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Previous papers<sup>1 2</sup> have described the accumulation of ammonia<sup>3</sup> by *Valonia macrophysa*, Kütz, from sea water containing very small concentrations of ammonium chloride. In such cases the gain in ammonia is accompanied by a loss of potassium.

In the present paper the exit of the accumulated ammonia into ammonia free sea water is discussed. This exit is accompanied by an intake of sodium or potassium. In weak light, sodium alone enters, in stronger light, potassium is also taken up.

The experiments were carried out in 4 stages.

*Stage I*—The cells were placed in rather dim light in sea water to which ammonium chloride had been added. During this stage they stored ammonia.

*Stage II*—The cells were placed in ammonia free water in dim light and the exit of ammonia was followed. Sodium was taken up.

*Stage III*—The exit of ammonia continued but in stronger light. Both sodium and potassium were taken up.

*Stage IV*—The cells were replaced in sea water to which ammonium chloride had been added. They again took up ammonia.

### EXPERIMENTAL

The experiments were carried out in Bermuda in the winter of 1936-37 and supplemented by some experiments in the summer of 1937<sup>4</sup>. A typical experiment will be described in detail.

<sup>1</sup> Cooper, W. C., Jr. and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 117.

<sup>2</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 301.

<sup>3</sup> The term ammonia as used in this paper means total ammonia: i.e.,  $\text{NH}_3 + \text{NH}_4\text{OH} + \text{NH}_4^+$ .

<sup>4</sup> The usual difficulties associated with the work on *Valonia* in the summer were

*Stage I*—A group of about 1000 cells (selected to exclude large and small ones) was exposed to sea water containing 0.0025 M  $\text{NH}_4\text{Cl}$  at pH 8.2, in a 4-gallon bottle. The bottle was suspended, immersed, in a long concrete tank, through which a rapid stream of sea water ran. In this way cooling and agitation was secured since the bottle swayed back and forth in the current and the cells in the bottle were gently stirred.

Two smaller groups of cells in smaller bottles were similarly disposed in the tank. These were used in measuring the volume change, and as in previous work it was assumed that the volume change of these cells was characteristic of the larger group.

The only illumination provided was the rather dim daylight present in the basement laboratory.

The cells were allowed to accumulate ammonia for 16 days.

*Stage II*—The cells were then transferred to a large glass aquarium in which a rapid stream of natural, nearly ammonia-free sea water was maintained. The aquarium was immersed to within a few inches of the top in the large concrete tank. Each group of "volume" cells was placed in a wide tube closed at each end by a wide mesh gauze. These were placed in the aquarium. During this stage the cells lost ammonia. This stage lasted 16 days.

*Stage III*—After 16 days the glass aquarium with the cells was transferred to a covered veranda with an eastern exposure. The illumination during the whole of this stage was bright daylight, but the cells were protected from direct sunlight by the lattice screens of the veranda. This stage lasted 10 days.

*Stage IV*—The few remaining cells were transferred to sea water containing 0.0025 M  $\text{NH}_4\text{Cl}$ , and were permitted to reaccumulate ammonia for 7 days.

At intervals during the first three stages, cells were removed and the sap extracted for analysis, for ammonia, potassium, sodium, and halide. At the same time the volumes of the "volume" groups were determined by the method described in a previous paper.<sup>5</sup> The potassium was determined by the micro-chloroplatinate method described in a previous paper,<sup>6</sup> using 0.1 ml samples, measured out by means of the washout pipette described by Pregl.<sup>7</sup> The sodium was determined by the Barber and Kolthoff<sup>8</sup> zinc uranyl acetate method, using 0.5 ml samples

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encountered, and several experiments were abandoned because of the high mortality during the accumulation stage. Eventually, by resorting to cooling, mortality was reduced below a reasonable limit and results were obtained, which, since they agree with those obtained in the winter when mortality is not a factor, must be regarded as significant.

<sup>5</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537.

<sup>6</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 967.

<sup>7</sup> Pregl, F., *Quantitative organic micro analysis*, first English edition, P. Blakiston's Son and Co., Philadelphia, 1930, p. 118.

<sup>8</sup> Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, 1928, 50, 1625, 1929, 61, 3233.

measured out with Pregl's pipette. Because of the relatively high concentration of potassium in the sap, double the usual amount of reagent was used, and the determinations were carried out at a temperature of 21–23 °C.<sup>9</sup>

In both the potassium and sodium analyses the sap was evaporated to dryness in platinum and ashed at about 300°C to remove organic material and ammonium which interfere with the potassium analysis.

Ammonia was determined by means of Nessler reagent,<sup>10</sup> using the Zeiss Pulfrich "step-photometer."

The sap samples, which varied in volume from 1 to 0.1 ml, were subjected to steam distillation in an apparatus similar to that described by Teorell,<sup>11</sup> and the ammonia was caught in 10 ml of 0.01 N H<sub>2</sub>SO<sub>4</sub> solution. Previously<sup>12</sup> when we used this method we failed to obtain a linear calibration curve at higher concentrations for the relationship between the "extinction coefficient" and the concentration of ammonia. In the present case this difficulty was overcome by increasing the quantity of Nessler's reagent used.

Halide was determined on 0.1 ml samples by electrometric titration with 0.04 M AgNO<sub>3</sub> using a silver electrode as the indicating electrode.

In order to average the natural differences among the cells, the minimum number taken for analysis was 40, and in a number of cases the sap was extracted separately in two equal lots and separate analyses were made on each, in order to get an idea of the natural variation.

The data for a typical experiment are given in Table I and Fig. 1. Where two analyses were made both are given and also the average. In the figure the results are expressed as moles  $\times 10^3$ ,  $\epsilon$ , concentration  $\times$  volume  $\times 10^3$ . The advantages of this have been discussed in a previous paper.<sup>2</sup>

The curves have been drawn free hand to give an approximate fit.

#### DISCUSSION OF RESULTS

As Fig. 1 shows, during Stage I ammonia entered the cell. The rate of gain of moles of total ammonia<sup>3</sup> was nearly linear. This point, however, will not be stressed unless confirmed by further experiment,

<sup>9</sup> At 15°C large amounts of potassium zinc uranyl acetate were precipitated along with the sodium compound.

<sup>10</sup> Nessler's reagent according to Fohn Wu (Fohn, O., and Wu, H. *J. Biol. Chem.*, 1919, 38, 81). As suggested by Fohn and Svedberg (Fohn, O., and Svedberg, A. *J. Biol. Chem.*, 1930, 88, 77) 2 drops of gum ghatti per 50 ml of the ammonia distillate were added before the addition of the Nessler reagent.

<sup>11</sup> Teorell, T. *Biochem. Z. Berlin*, 1932, 248, 246.

<sup>12</sup> Jacques, A. G. *Proc. Nat. Acad. Sci.*, 1935, 21, 488.

TABLE I  
*The Accumulation and Exit of Ammonia in Valonia Analyses of Sap*

Stage	Days	Volume ml	Molar concentration in the sap					Moles $\times 10^3$		
			K	Na	Total ammonia	K + Na + ammonia	Halide	K	Na	Total ammonia
I Accumulation of ammonia	0	2 46	0 5127	0 1381	0 00039	0 6439	0 6360	1 249	0 3336	0 00089
			0 5031	0 1330	0 00032		0 6200			
	4	2 50	Av 0 5079	Av 0 1356	Av 0 00036		Av 0 6280			
	10	2 49	0 4914	0 1186	0 0287	0 6387	0 6260	1 229	0 2965	0 0718
	16	2 60	0 4763	0 1128	0 0543	0 6434	0 6320	1 186	0 2809	0 1352
II Exit of ammonia in dim light			0 4417	0 1135	0 0871	0 6395	0 6361	1 155	0 2982	0 2098
			0 4465	0 1158	0 0742					
			Av 0 4441	Av 0 1147	Av 0 0807					
	0	2 60	0 4441	0 1147	0 0807	0 6395	0 6361	1 155	0 2982	0 2098
	4	2 62	0 4462	0 1224	0 0729	0 6415	0 6234	1 169	0 3207	0 1910
	7	2 61	0 4419	0 1254	0 0736	0 6409	0 6260	1 153	0 3273	0 1921
	10	2 59	0 4394	0 1305	0 0629	0 6368	0 6200	1 141	0 3421	0 1665
			0 4413	0 1336	0 0657					
			Av 0 4404	Av 0 1321	Av 0 0643					
	12	2 65	0 4474	0 1347	0 0586	0 6407	0 6236	1 186	0 3570	0 1553
	14	2 64	0 4342	0 1469	0 0486	0 6347	0 6290	1 152	0 3920	0 1320
			0 4381	0 1501	0 0514					
	16	2 63	Av 0 4362	Av 0 1485	Av 0 0500	0 6395	0 6244	1 170	0 3966	0 1152
			0 4449	0 1508	0 0438					

III Exit of ammonia in bright light	0 (16)*	2 63	0 449	0 1508	0 0438	0 6395	0 6244	1 170	0 3966	0 1152
	2 (18)	2 70	0 4216	0 1842	0 0305	0 6358	0 6414	1 138	0 4968	0 0824
			0 4210	0 1838						
	4 (20)	2 75	Av 0 4213	Av 0 1840	0 0138	0 6324	0 6262	1 175	0 5258	0 0380
IV Re accumulation of ammonia	6 (22)	2 78	0 4274	0 1912	0 00593	0 6396	0 6346	1 232	0 5299	0 0165
	8 (24)	2 75	0 4431	0 1906	0 00172	0 6470	0 6262	1 228	0 5464	0 00473
	10 (26)	2 75	0 4466	0 1987	0 00072	0 6532	0 6320	1 235	0 5596	0 00198
			0 4490	0 2035	0 00072	0 6532	0 6320			
	0		0 4490	0 2035	0 00072	0 6532	0 6392			
	7†		0 2905	0 2039	0 1345	0 6289				

\* Bracketed figures are the number of days since the start of the exit of ammonia

† 5 days in bright light and 2 days in darkness

although it agrees with results previously obtained<sup>13</sup> At the same time there was a loss of moles of potassium, also in agreement with previous results It seems not unlikely, in view of the scattering of

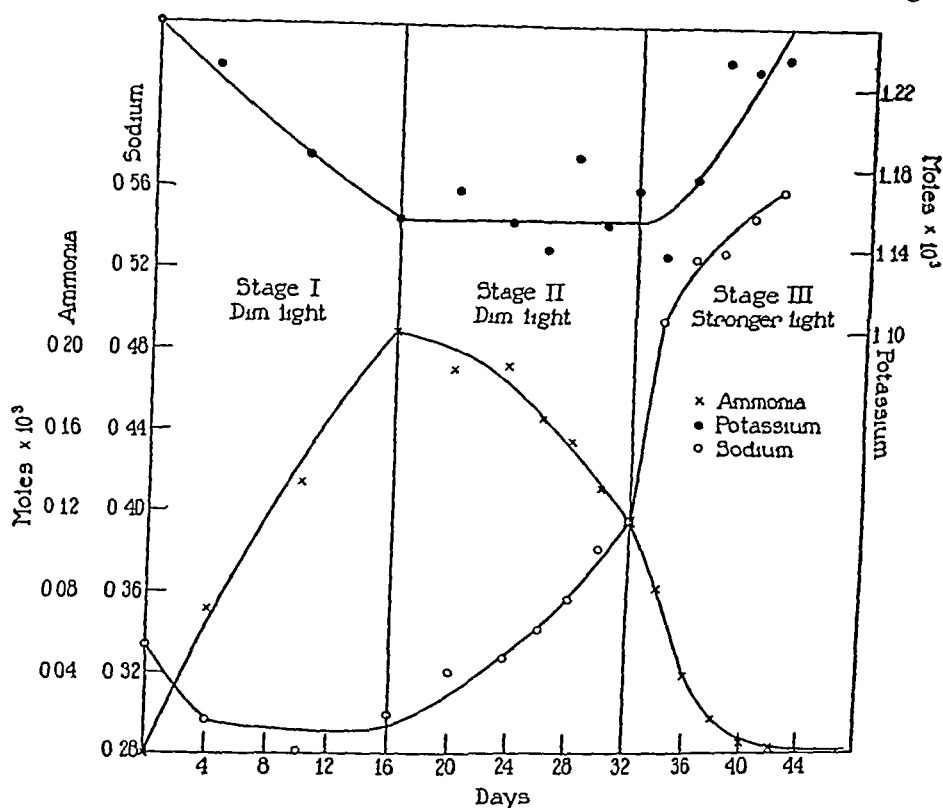


FIG 1 Curves showing entrance and exit of ammonia (X), potassium (●), and sodium (○) in *Valonia*

*Stage I*—The cells were in dim light in sea water containing 0.0025 M ammonia ( $\text{NH}_3 + \text{NH}_4\text{OH} + \text{NH}_4^+$ ) Ammonia increased, sodium and potassium came out

*Stage II*—The cells were in dim light in normal sea water (nearly free from ammonia) Ammonia came out and sodium went in, potassium remained more or less constant

*Stage III*—The cells were in stronger light in normal sea water Ammonia came out, sodium and potassium went in

the potassium points in Stages II and III that the regularity of the curve is fortuitous However, there is no reason to doubt that potas-

<sup>13</sup> Jacques, A G, and Osterhout, W J V, *J Gen Physiol*, 1930-31, 14, 301 Up to 20 days the rate of gain of moles of ammonia was approximately linear, thereafter it decreased



sium came out. In this stage also there was a loss of sodium from the sap, and this appears to have taken place chiefly in the first 4 days.

Although the measurements were somewhat irregular, there seems also to have been some increase in volume during this stage. This perhaps represents the stretching of the cell wall which occurs when a rapidly penetrating substance, such as ammonia, is present in the sea water. Evidence of this stretching was seen in the very noticeable increase in turgidity.

From the table it appears that during Stage I the sap lost  $0.09 \times 10^{-3}$  mole of potassium and gained  $0.21 \times 10^{-3}$  mole of ammonia.

During Stage II in which the cells were exposed to a flow of normal, nearly ammonia free sea water, in dim light, there was no definite change in the number of moles of potassium in the sap. The points are somewhat irregularly scattered about the curve, and the trend, if there is any, is only slightly upwards.

In this stage ammonia passed out of the cell. The rate of exit was slower than the rate of entrance during Stage I. In the first 16 days of exit the cell lost about half the ammonia it had accumulated in the previous 16 days, so that the rate of exit was roughly half the rate of entrance. In other experiments relative rates of the same order were observed.

During Stage II there was no significant change in the moles of potassium, but the sap lost  $0.095 \times 10^{-3}$  mole of ammonia and gained  $0.098 \times 10^{-3}$  mole of sodium.

During Stage III in which the cells were exposed to running ammonia free sea water in bright daylight, as Fig. 1 shows, the potassium moles increased. The points are somewhat scattered, but the fact of the increase is not in doubt.

The exit of ammonia continued during this stage, at first at a markedly greater rate, but towards the end of the experiment the rate fell off as the concentration of the ammonia in the cell decreased.

The behavior of sodium in this stage was roughly the inverse of the behavior of ammonia, but the increase in the rate of entrance of sodium was greater than the increase in the rate of ammonia exit, and the subsequent falling off in the rate of entrance of sodium was not nearly so great as the falling off in the rate of exit of ammonia. Consequently the sap as a whole gained more moles of sodium than it lost of ammonia.

While the cells lost  $0.11 \times 10^{-3}$  mole of total ammonia they gained  $0.16 \times 10^{-3}$  mole of sodium and about  $0.07 \times 10^{-3}$  mole of potassium or  $0.23 \times 10^{-3}$  mole in all, a net gain of  $0.12 \times 10^{-3}$  mole. About one half of this is accounted for by the increase in volume and the remainder probably represents the increase in total concentration which is usually observed when the cells are taking in electrolytes in daylight.

The shape of the ammonia exit curve indicates that ultimately the exit would cease at a very low internal concentration of the same order as that found in naturally growing cells. This is somewhat variable but over a period of years we have found it to be as high as  $0.0006 \text{ M}^1$  and as low as  $0.0002 \text{ M}^{11}$ . In any one collection kept under similar conditions, the variation of samples from uniform groups of 10 to 20 cells is usually much less than this. The source of the ammonia is unknown. Possibly, however, it is the result of "accumulation," since natural sea water contains traces of ammonia.<sup>16</sup>

If the pH of the sap at the end of Stage III was 6.0 the concentration of undissociated ammonia in it would have been  $1.44 \times 10^{-7}$ . And if the external pH were 8.2, the concentration of undissociated ammonia in the sea water, taking  $0.000002 \text{ M}$  as the average for total ammonia, would have been  $0.67 \times 10^{-7}$ . But since the cells were illuminated during the daylight hours the external pH was probably greater on the average than 8.2. If it was 9.4 during the day and 8.2 during the dark the average would be 8.5 and the external concentration of undissociated ammonia would be  $1.29 \times 10^{-7}$ . These calculations are only approximate but they indicate that the concentration of ammonia naturally present in cells is of the right order of magnitude for accumulation of total ammonia (with undissociated ammonia approximately equal outside and inside, possibly going in during the day and out at night).

It is possible, however, that the ammonia naturally present in the cells is the result of metabolism. Unpublished results of the author indicate that there is a significant concentration of nitrate in the sap and the reduction of this at the protoplasmic surface may produce ammonia in the sap. In that case ammonia might escape into the sea water until the "accumulation equilibrium" indicated above was established.

<sup>14</sup> Author's unpublished results.

<sup>15</sup> No analyses are available for Bermuda sea water. See footnote 19.

Let us now consider the factors determining the movement of ammonia, potassium, and sodium during the experiment

In Stage I the external pH was 8.2 (average for sea water in Bermuda) and the concentration in sea water of total ammonia<sup>3</sup> was  $0.0025\text{ M}$ <sup>16</sup>. For convenience,  $pK_{ab}$  is taken as 9.66<sup>1</sup> for both sap and sea water, since their ionic strengths are not far apart<sup>18</sup>. From these values the undissociated ammonia in the sea water is 3.35 per cent of the total ammonia or  $8.38 \times 10^{-5}\text{ M}$ . And if the pH of the sap was 6.0, corresponding to an undissociated fraction of 0.02 per cent, the concentration of undissociated ammonia in the sap at the end of Stage I was  $1.61 \times 10^{-5}$  or about 20 per cent of the outside concentration of undissociated ammonia. Under these circumstances it is not surprising that the back diffusion was unimportant as evidenced by the slight curvature of the ammonia accumulation curve towards the time axis.

In an experiment reported previously the curve of ammonia accumulation likewise was nearly linear up to 20 days even though the concentration of ammonia outside was only  $0.001\text{ M}$  and the internal concentration of ammonia was greater than was attained in the present experiment. However in a former experiment, accumulation took place in daylight so that the external pH, due to photosynthetic removal of carbon dioxide, may have been much greater in the layer of sea water next to the protoplasm. At pH 9, which may easily be possible in the vicinity of the cell, during photosynthesis, the undissociated fraction of ammonia would be about 18 per cent, so that the concentration of undissociated ammonia in the sea water may have been  $1.8 \times 10^{-4}\text{ M}$ . At the end of 20 days the total ammonia in the sap was  $0.1119\text{ M}$  and assuming the pH to be 6, the corresponding concentration of undissociated ammonia  $2.2 \times 10^{-5}$ . So in this case also back diffusion may have been unimportant.

Now the rate of entrance of ammonia is considered to be proportional to the gradient of concentration of undissociated ammonia in the non aqueous protoplasmic surface layer and provided the partition coefficients between the sea water and protoplasmic surface and

<sup>16</sup> Neglecting the small concentration of ammonia normally present in the cells

<sup>17</sup> The assumptions and steps leading to the adoption of this value have been given in another paper (Jacques A. G., *J. Gen. Physiol.* 1935-36 19, 403-409) and need not be repeated here

<sup>18</sup> According to Zscheile (Zscheile, F. P. Jr *Protoplasma*, 1930, 11, 481) the ionic strength of Bermuda sea water is 0.72 and of *Valonia* sap 0.67. The latter, corrected for a misprint in the concentration of calcium in the sap becomes 0.61

sap and protoplasmic surface are approximately equal, which seems not unlikely, the rate is also proportional to the difference of concentration of undissociated ammonia between the sap and sea water

In Stage II during the exit of ammonia the average internal concentration of undissociated ammonia (assuming the pH of the sap to be 6) was  $1.22 \times 10^{-5}$  and since the sea water is nearly ammonia-free, the back (inward) diffusion must have been very small indeed

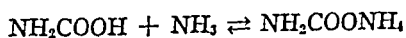
We have no figures for the concentration of ammonia in Bermuda sea water but using the average value of the analyses made elsewhere by others,<sup>19</sup> viz 0.000002 M, we get for the concentration of undissociated ammonia in normal sea water  $6.6 \times 10^{-8}$ . If now we compare the inward concentration gradient in Stage I with the outward concentration gradient in Stage II we find that the former is about 6 times the latter, but the rate was only about twice as great. But it seems probable that the entrance of ammonia increases the pH of the sap. Such changes have been observed experimentally<sup>1</sup> and from a theoretical standpoint we should expect, as ammonia accumulates, that the ammonium bicarbonate equilibrium would dominate the buffer situation instead of the potassium (and sodium) bicarbonate equilibrium. Under the simplest assumptions the midpoint pH of this system would be given by the relationship

$$\text{pH} = \frac{1}{2}\text{pK}_{\text{H}_2\text{O}} + \frac{1}{2}\text{pK}'_a - \frac{1}{2}\text{pK}'_b$$

whence  $\text{pH} = 7.84$ <sup>20</sup>. This value, however, may be too high since, as Wegscheide<sup>21</sup> has shown, the ammonium bicarbonate equilibrium may be modified by the presence of ammonium carbamate, produced by the reversible reactions



and



<sup>19</sup> Robinson, R. J., and Wirth, H. E., *J. conseil internat. exploration mer*, 1934, 9 (1), 15, 193, 1934, 9 (2), 187, found for the surface waters of Puget Sound and for the Pacific Ocean off Vancouver Island 0.000012 M, and Serwell, H. R., *Ecology*, 1931, 12, 485, found for the Atlantic Ocean off Mount Desert, Maine, 0.000003 M.

<sup>20</sup>  $\text{pK}_{\text{H}_2\text{O}} = 14.00$ ,  $\text{pK}'_a = 6.06$ ,  $\text{pK}'_b = 4.34$ . The reason for the choice of these values has been given in a previous paper. See footnote 17.

<sup>21</sup> Wegscheide, R., *Monatsh. Chem.*, 1916, 37, 425.

The effect of this factor depends (a) on the concentration of carbamate and (b) on the dissociation constant of carbamic acid. According to Faurholt's<sup>22</sup> curves at pH 6.0 the fraction of the carbonic compounds, viz.  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{--}$ , and  $\text{NH}_2\text{COO}^-$  present as carbamate is extremely small but at pH 7.84 it is about 5 per cent of the total. Also according to Faurholt the ionization constant of carbamic acid must be greater than  $10^{-7}$  and is probably much greater than this. It has, however, owing to experimental difficulties not been measured satisfactorily. The most we can say therefore is that in all probability the formation of carbamate as the pH of the sap rises will tend to limit the amount of rise. It should be pointed out also that the formation of ammonium carbamate would temporarily immobilize part of the ammonia entering the cell. But as the ammonia is removed the pH of the sap will drop and the carbamate will be decomposed. Hence the carbamate can affect only the rate of exit and not the equilibrium between the sap and sea water.

The effect of an increase in the pH of the sap during accumulation will be to increase the back pressure and so decrease the rate of entrance of ammonia. On this account the estimate that the inward gradient during accumulation was 6 times the outward gradient during exit may have been too high. There remains also to be considered the possibility that exit takes place when the pH of the sap is higher than 6.0. If, as suggested above, the ammonium bicarbonate equilibrium dominates the pH situation, as long as there is much ammonia in the sap, we should expect the pH to be greater than 6.0. Because of the uncertainty due to the carbamate we can only estimate roughly the extent of the rise to be expected. If, however, the internal pH increased to 6.5, the average concentration of undissociated ammonia during the first exit stage (Stage II) would have been about  $0.042 \times 10^{-5} \text{ M}$ , that is, about one half the external concentration of undissociated ammonia during the accumulation stage.

These approximate calculations indicate that the inward and outward rates may well be proportional to the gradient of undissociated ammonia.

Let us now consider the behavior of sodium. During the accumulation of ammonia in all previous experiments,<sup>2-4</sup> which were carried on in bright light, we observed that sodium as well as ammonia entered the cell. This is to be expected on theoretical grounds since in bright

<sup>22</sup> Faurholt, C., *J. chim. physique*, 1925, 22, 1

<sup>3</sup> Also unpublished results

light the increase in pH of the sap produced by ammonia could scarcely be great enough to abolish the difference of chemical potential between sodium hydroxide outside and inside. It is well known that in bright light the pH of the sea water adjacent to the cell can be raised to 9.6 or higher by photosynthesis.

In the present experiments performed in dim light (Stage I) the concentration of sodium remained approximately constant in all experiments except in that shown in Fig. 1 where the curve indicates that a loss of sodium occurred during the first few days and that sodium thereafter remained approximately constant. This suggests that the initial point is too high and that the sodium actually remained approximately constant during the entrance of ammonia. If the pH values inside and outside were such as to make sodium come out it would seem that more ammonia entered than would be expected.

When the illumination is low and photosynthesis is reduced there is a constant escape of carbon dioxide from the cell and this may well reduce the pH of the layer of sea water adjacent to the protoplasm below 8.0. We cannot determine the extent of these pH changes, but at any rate we can say that the rate of entrance of sodium should be much less when the light is diminished.

As Steward<sup>24</sup> has recently affirmed his belief that the loss of potassium and the gain of sodium during accumulation of ammonia indicate injury, it seems desirable to point out that sodium did not enter in dim light and on theoretical grounds might not be expected to do so.

The question remains whether in the previous experiments there was injury in the sense that in each analysis a few cells injured, so as

<sup>24</sup> Steward, F. C., *Nature*, 1935, 135, 553. More recently Steward and Martin (Steward, F. C., and Martin, J. C., *Carnegie Institution of Washington, Pub. No. 475*, 1937, 87) have amplified the view that the changes in the concentration of potassium and sodium during the entrance of ammonia are the result of injury, chiefly on the grounds that (a) injury would cause changes in the direction observed, (b) in their experiments in the Tortugas in the summer the cells were obviously injured. Suffice it to say that the first argument is effectively met by the present results which show a loss of both potassium and sodium, and the second by the fact that the interesting series of changes in the appearance of the cells described by Steward and Martin for *Valonia ventricosa* of Tortugas, in the summer, were completely absent in the case of *Valonia macrophysa* in Bermuda in winter.

to be more permeable to all the constituents of the sea water, may have been included. Reasons for rejecting this possibility have already been given. These still seem valid to us.<sup>5</sup>

It is evident that the pH of the aqueous solutions in contact with the protoplasm may be very different from that in the main body of the solutions. For example, when ammonia is entering the pH of the layer of sap in contact with the protoplasm must be considerably higher than the average pH of the sap as a whole.

We have no way of measuring the pH of the thin aqueous layers immediately in contact with the protoplasm, but we do know (a) that the entrance of ammonia can raise the pH of the sap, and (b) that in bright light the photosynthetic removal of carbon dioxide can raise the pH of the sea water. If we assume that the pH changes which have been demonstrated to take place in the body of the sap and sea water may take place much more rapidly and to a greater extent in the thin aqueous layers adjacent to the protoplasm, we can explain why potassium came out in Stage I, why sodium alone entered in Stage II, and why both sodium and potassium entered in Stage III.

From thermodynamic considerations the direction of movement of KOH through the non aqueous layers of the protoplasm will depend on the difference of free energy of KOH between the sap and sea water. This is given by the equation

$$\Delta F = RT \ln (K)_o(OH) - RT \ln (K)_i(OH)$$

where *o* and *i* refer to the sea water (outside) and the sap (inside) respectively, and round brackets represent activities. (OH) is known with sufficient accuracy from pH measurements. For potassium it is more convenient to substitute [K] for (K), where the square brackets indicate concentration. This may be done without objection, because the ionic strengths of the sap and sea water are nearly alike and hence the activity coefficient of potassium can be taken as the same in sap and sea water.<sup>6</sup>

<sup>5</sup> Osterhout, W J V, *Nature*, 1935, 136, 1034

<sup>6</sup> As explained in a previous paper (Jacques A. G., *J Gen Physiol*, 1936-37, 20, 737) for convenience we assume that single ion activity coefficients have real existence.

In place of  $\Delta F$ , we can use for the purposes of determining the direction of movement, the difference  $[K]_o(OH)_o$  and  $[K]_i(OH)_i$ . Similar considerations apply to other molecules passing through the protoplasm, such as  $NaOH$ ,  $NaCl$ , etc

In Stage I it is easy to see why potassium should leave the cell when ammonia goes in, for the ammonia raises the pH of the sap, particularly in the layer in contact with the protoplasm, and presumably makes  $[K]_i(OH)_i > [K]_o(OH)_o$ .

In Stage II, when ammonia leaves the cell, we suppose that it lowers the pH of the layer of the sap adjacent to the protoplasm sufficiently to make  $[K]_i(OH)_i$  approximately equal to  $[K]_o(OH)_o$ . During this stage, however,  $[Na]_o(OH)_o$  is greater than  $[Na]_i(OH)_i$ , and sodium continues to enter.

In Stage III, in brighter light, we suppose that photosynthesis raises the pH of the layer of sea water adjacent to the protoplasm. This causes  $[K]_o(OH)_o$  to become greater than  $[K]_i(OH)_i$ , and potassium and sodium enter to replace the ammonia leaving the cell. The process is aided by the decrease of ammonia in the sap which results in a decrease of the pH of the sap as the ammonium bicarbonate buffer system is replaced by the sodium and potassium bicarbonate buffer system.

It may be remarked in passing that there was no suggestion of injury in the later stages any more than in Stage I (p. 676). The fact that in Stage II the ammonium was actually replaced entirely by sodium, must not be regarded as evidence of injury in the sense of an increase in the permeability of the protoplasm to all constituents of the sea water and sap. There was no appearance of injury<sup>27</sup> in the cells, sulfate was absent from the sap, halide did not decrease, potassium did not decrease but in the last part of the experiment actually increased. All this indicates that such injury had not occurred.

Further evidence that the protoplasm has not been drastically altered by the ammonia treatment is found in the fact that after the removal of nearly all the ammonia the cells were again able to accumulate ammonia from sea water containing ammonia. There was a loss

<sup>27</sup> Regarding signs of injury see Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 967.



of potassium, as Table I shows<sup>28</sup> It is interesting to speculate on the possibility that the cells might again replace most of the ammonia by sodium on removal to ammonia free sea water In such a case the sodium concentration might be nearly 3 times what it was at the start, and the potassium concentration only about 60 per cent of its initial value, and the ratio of K/Na would be less than 1 There remains the possibility that the ratio might be reduced even more Such cells might have very different electrical properties from normal *Valonia* cells This remains a subject for future investigation

For convenience we have hitherto spoken of the entrance of potassium and sodium in the form of hydrates There is no objection to this so long as we are merely considering the direction of movement of different substances or different rates of the same substance under different conditions, but when we compare the rates of entrance of sodium and potassium other considerations come in, as will appear from the following

The situation when the cells are taking in electrolytes from normal sea water may be as follows

$$\begin{array}{llll} \text{pH} = 6.0 & \text{p(OH)}_i = 8.0 & \text{pH}_o = 8.2 & \text{p(OH)}_o = 5.8 \\ K_i = 0.5 \text{ M} & K_o = 0.012 \text{ M} & \text{Na} = 0.12 \text{ M} & \text{Na}_o = 0.5 \text{ M}^{29} \end{array}$$

whence

$$\begin{array}{ll} [\text{K}](\text{OH}) = 10^{-7.72} & [\text{Na}](\text{OH}) = 10^{-4.22} \\ [\text{K}](\text{OH})_i = 10^{-4.29} & [\text{Na}](\text{OH}) = 10^{-5.22} \end{array}$$

It is probable that the bases move through the protoplasm as  $M\text{X}$ , where  $\text{X}$  is the anion of a weak acid elaborated by the protoplasm As Osterhout<sup>30</sup> has pointed out, at the interface between an aqueous phase and a non aqueous phase there are unstirred layers in which the solutes move only by the relatively slow process of diffusion and that in the regions where the two phases are immediately in contact the solutes are in equilibrium across the boundary This equilibrium is

<sup>28</sup> The fact that the rate of accumulation of ammonia was much greater in this stage is not necessarily significant, since the conditions of accumulation were different

<sup>29</sup> These are average values

<sup>30</sup> Osterhout W J V, *J Gen Physiol* 1932-33, 16, 529

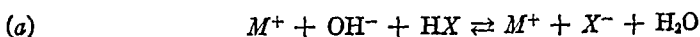
expressed by the partition coefficient,  $S$ , which is the ratio of the concentration of a solute in the non-aqueous phase to its concentration in the aqueous phase

In the cell there are two pairs of unstirred layers, one at the sea water-protoplasm, and the other at the sap-protoplasm interface. Now we may safely assume that there are no gradients of concentration for  $M^+$  or  $OH^-$  in the sea water or sap unstirred layers. This will not be true for  $X^-$  or  $HX$  since the concentration of  $X^-$  and  $HX$  in the sea water must be taken as infinitely small. However, the gradients for these substances may be numerically very small indeed for the reason that  $MX$  and  $HX$  have extremely high partition coefficients. In such a case the loss of  $MX$  and  $HX$  to the sea water may be very slow indeed.

The force of the above discussion is that  $M^+$  and  $OH^-$  are known in the sap and in the sea water, and if there are no concentration gradients in the unstirred layers we also know their concentrations immediately at the protoplasmic interfaces.

Neither  $MX$  nor  $HX$  is known in any of the regions under discussion, but these terms will be eliminated ultimately from the rate equations. Until this is done, however, we shall speak of  $MX_{op}$  and  $HX_{op}$  where  $op$  refers to the thin region in the sea water unstirred layer, where the solutes are in equilibrium with the protoplasm. For the sap-protoplasm interface we have a corresponding pair,  $MX_p$  and  $HX_p$ .

Let us suppose that  $MX$  is formed in the sea water by the reaction



whence

$$(b) \quad \frac{f_M^o f_{OH}^o f_{HX}^{op}}{(f_{\pm}^{op})_{MX}^2} \frac{[M]_o [OH]_o [HX]_{op}}{[M]_o [X]_{op}} = k_{hyd} *$$

\*  $f_M^o f_{OH}^o$  is substituted for the more accurate value  $(f_{\pm}^o)^2$  for practical reasons (see Jacques, A. G., *J. Gen. Physiol.*, 1936-37, 20, 747)

where  $f^o$  is the activity coefficient in the sea water,  $f^{op}$  is the activity coefficient in the thin region in the aqueous unstirred layer, and  $k_{hyd}$  is the hydrolysis constant, and since  $HX$  is a weak acid,  $f_{HX}^{op}$  may be taken as unity. But

$$(c) \quad (f_{\pm}^{op})_{MX}^2 [M]_o [X]_{op} = k_{dis} f_M^{op} [MX]_{op}$$

where  $k_{dis}$  is the classical dissociation constant. Whence by substitution in (b) and rearrangement,

$$(d) \quad [HX]_{op} [M]_o f_{OH}^o [OH]_o = \frac{k_{hyd} k_{dis} f_{MX}^{op} [MX]_{op}}{f_M^o}$$

But

$$(e) \quad S_{op} [MX]_{op} = [MX]_p$$

where the subscript  $po$  refers to the unstirred non aqueous layer in the protoplasm immediately in contact with the external aqueous phase, and  $S_p$  is the partition coefficient. For the sap we have similar relationships,

$$(f) \quad [HX]_p [M]_i f_{OH}^i [OH]_i = \frac{k_{hyd} k_{dis} f_{MX}^i [MX]_p}{f_M^i}$$

and

$$(g) \quad S_p [MX]_p = [MX]_p$$

Now disregarding restriction due to the cell wall the rate of gain of  $M$  by the sap should be given by

$$(h) \quad \frac{dM}{dt} = D^{MX} (M Y_{ps} - M Y_{pi}) = D^{MX} (S_p [MY]_p - S_{ip} [MX]_{ip})$$

where  $D^{MX}$  is the rate of flux through the protoplasm under standard conditions, which is analogous to the diffusion constant<sup>31</sup>

Now comparing equations (d) and (f)

$$f_{MX}^p = f_{MX}^i \quad \text{and} \quad f_M = f_M^i$$

since the ionic strengths of the sap and the sea water are close to each other, and

$$f_{OH}[OH] = (OH) \quad \text{and} \quad f_{OH}^i [OH]_i = (OH)_i$$

(where curved brackets refer to activities and square brackets to concentrations)

Both of these are known if the pH of the sea water and sap are known. Then

$$\frac{f_{MX}^p k_{hyd} k_{dis}}{f_M} = \frac{f_{MX}^i k_{hyd} k_{dis}}{f_M^i} = k_{coll}$$

where  $k_{coll}$  is a collection of all the constants

Now  $S_p$  may be taken as equal to  $S_{ip}$  and then

$$(i) \quad \frac{dM}{dt} = \frac{D^{MX} S}{k_{coll}} \{ ([HX]_{ps} [M] (OH)) - ([HX]_{ip} [M]_i (OH)_i) \}$$

Now if  $HX$  is elaborated equally throughout the protoplasm so that  $[HX]_p = [HX]_{ps}$  then provided that  $S_{HX}^p = S_{HX}^i$  we have  $[HX]_p = [HX]_{ip}$ . For the mo-

<sup>31</sup> The flux through the protoplasm depends to some extent on the aqueous phase between the non aqueous surface layers (these two layers are treated as one in the present discussion). But the influence of the aqueous phase is probably very small. It has been shown in a previous paper (Jacques, A G *J Gen Physiol*, 1936-37, 20, 737) that the diffusion through the non aqueous layers is more than a million times slower than through an aqueous phase.

ment we shall make the assumption that this is true, and that these concentrations are constant

Applying equation (1) to K and Na entrance,  $k_{ent}$  for both may be taken as equal, since both are strong bases, and the ratio of the rates of entrance of K to Na is then given by

$$(k) \quad \frac{\left(\frac{dM}{dt}\right)^K}{\left(\frac{dM}{dt}\right)^{Na}} = \frac{D^K S^K}{D^{Na} S^{Na}} \frac{[K]_o(OH)_o - [K]_i(OH)_i}{[Na]_o(OH)_o - [Na]_i(OH)_i}$$

For convenience we may put  $DS - k_{ent} = P$ , the permeability constant. The ratio of the concentration of potassium to sodium in the sap must be the ratio of the average rate of entrance and on this basis Osterhout<sup>32</sup> has calculated that the permeability to KOH is 331 times as great as to NaOH. With the values assumed in this paper, the ratio  $P_{KOH} - P_{NaOH}$  becomes 236. The exact value, which will vary somewhat according to the analysis of sap, is unimportant. In any case it indicates a very large difference between the permeability of the protoplasm to potassium and to sodium. This difference is probably due, as Osterhout points out, to a large difference in the partition coefficients.

These considerations serve to show how the relative rates of entrance of sodium and potassium are determined. The fact that the ratio of K/Na in the sap is variable indicates that the relative rates of entrance may vary from cell to cell. This raises the question whether they may not change for a single cell during its absorption of electrolytes. On the whole the evidence indicates such changes. Thus, as pointed out previously,<sup>2</sup> the ratio K/Na for small actively growing cells is greater than in large mature cells from the same collection. This might be expected on the ground that the more active the metabolism and the greater the production of carbon dioxide the lower will be the internal pH. A rise of internal pH will stop the entrance of potassium long before it stops that of sodium. Steward and Martin<sup>33</sup> found that naturally growing, well illuminated cells had a greater proportion of potassium than poorly illuminated ones. The cause of the change need not be an increase in the permeability of the protoplasm. Thus if the pH outside the protoplasm increases as the result of photosynthesis without a change in the internal pH, as

<sup>32</sup> Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 991.

<sup>33</sup> See reference 24.

found by Crozier,<sup>34</sup> and if the value 236 for  $P_{\text{KOH}} - P_{\text{NaOH}}$  given above is acceptable, we find that when the external pH increases from 8.2 to 8.5, using equation (k),  $K/Na$  increases at the steady state from  $0.50 - 0.12 = 4.2$  to  $0.515 - 0.105 = 4.9$ , and at pH 9.0 to  $0.523 - 0.097 = 5.4$ . Such changes in the pH of the sea water in contact with the protoplasm appear to us to be quite possible in well illuminated cells.

We are now in a position to take up the question, Why does sodium enter more rapidly when ammonia is coming out in Stage II than under normal conditions?

From the foregoing we derive the following equation for the rate of entrance of sodium as  $NaX$ ,

$$(l) \quad \frac{dNa}{dt} = DNa \{ S_{NaX}^{NaX} [NaX]_o - S_{NaX} [NaX]_i \}$$

We should expect sodium and potassium to enter as long as the products  $(K)_o(OH)_o$  and  $(Na)_o(OH)_o$  are greater outside than inside. But we find that when growth slows down and the entrance of water is lessened the entrance of sodium and potassium is also lessened. The total halide in the sap, which in the present experiments is a measure of the total electrolyte present,<sup>35</sup> tends to remain approximately constant. This suggests that when the concentration of halide in the sap reaches a certain value, which may be called for convenience the normal concentration, the penetration of electrolyte is checked until the absorption of water lowers the concentration enough to permit further entrance. It might seem that a regulatory mechanism existed for this purpose. It should be noted, however, that in models where we see no special regulatory mechanism, water and electrolyte enter in a fixed ratio so that the concentration of electrolyte in the artificial sap remains approximately constant.<sup>36</sup> A regulatory mechanism might take various forms. As equation (l)

<sup>34</sup> Crozier, W. J. *J. Gen. Physiol.*, 1918-19, 1, 581. See also, Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1933-34, 17, 727.

<sup>35</sup> Sodium, potassium, and ammonium appear in the sap almost wholly as halides. There is a little nitrate and a little bicarbonate but they may be neglected in the present discussion.

<sup>36</sup> Osterhout, W. J. V., and Stanley, W. M. *J. Gen. Physiol.*, 1931-32, 16, 667.

indicates, the rate of entrance of sodium appears to depend on (1) the concentration gradient of a sodium compound  $\text{NaX}$  in the protoplasm, (2) its partition coefficients between the aqueous and non-aqueous phases, and (3) its rate of movement,  $D$ , through the protoplasm under standard conditions

It seems possible that when the concentration of halide in the sap rises above the normal, the inner non-aqueous protoplasmic surface layer becomes sufficiently dehydrated to suffer a marked increase of viscosity and so to lower the value of  $D$ .

Other factors may assist in checking the entrance of electrolytes under these conditions. For example, the dehydration may also affect the partition coefficient of  $\text{NaX}$  at the inner non-aqueous protoplasmic surface in such a way as to check entrance of sodium. Thus if  $\text{NaX}$  is more soluble in the non-aqueous protoplasmic surface than in water, the removal of water may increase the solubility of  $\text{NaX}$  and so increase  $S_{i,p}^{\text{NaX}}$ . This will decrease the rate of entrance of  $\text{NaX}$  and hence of water.

The faster ammonia comes out of the cell the faster electrolytes can enter without exceeding the normal concentration of halide. In Stage II, we suppose that potassium cannot enter because  $[\text{K}]_i(\text{OH})_i$  is approximately equal to  $[\text{K}]_o(\text{OH})_o$  but sodium can enter and will do so more rapidly than usual because ammonia is coming out.

This will be discussed more fully in another paper<sup>37</sup>

#### SUMMARY

Experiments on *Valonia* were carried out as follows

*Stage I*—Cells in dim light accumulated 0.08 M ammonia ( $\text{NH}_3 + \text{NH}_4\text{OH} + \text{NH}_4^+$ ) from sea water containing 0.0025 M ammonia (but the concentration of undissociated ammonia appeared to remain less inside than outside). Potassium came out.

*Stage II*—Cells in dim light in nearly ammonia-free normal sea water lost ammonia which was replaced by sodium entering from the sea water. Potassium in the sap remained practically constant.

*Stage III*—The cells were placed in stronger light where the loss of ammonia continued and potassium entered. Sodium entered more rapidly than in Stage II.

<sup>37</sup> Unpublished.

*Stage IV* — Cells transferred to sea water containing 0.0025 M ammonia again accumulated ammonia up to 0.1345 M

The results in general harmonize with the view that the direction of movement of a base  $M$  through the protoplasm depends on the difference of the activity products  $(M)(OH)_o$  and  $(M)(OH)_i$ , where the subscripts  $o$  and  $i$  refer to sea water and sap respectively

On this basis, if the entrance of ammonia raised the internal concentration of OH sufficiently in Stage I potassium should come out in Stage I, as actually happened. The behavior of sodium is in doubt.

If the internal pH in Stage II were sufficiently high sodium should enter but not potassium. This was actually found.

In Stage III, if we suppose that the effect of stronger light is to increase the external pH (by photosynthesis) more than the internal pH (as found by Crozier) we can understand why potassium entered, because such an increase in pH could readily make the external value of  $(K)(OH)$  greater than the internal. This would also explain why sodium entered more rapidly than in Stage II.

When ammonia is coming out of the cell, sodium and potassium may enter more rapidly than usual without raising the internal concentration of halide above a certain critical value at which entrance appears to be checked.





# THE ACCUMULATION OF ELECTROLYTES

## X ACCUMULATION OF IODINE BY HALICYSTIS AND VALONIA

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Owing to its rôle in the thyroid, the accumulation of iodine has aroused especial interest

Evidently we cannot understand how any sort of accumulation comes about unless we know what happens inside the living cell during the process. Such information can be obtained by using the very large multinucleate cells of certain marine algae (*Halicystis* and *Valonia*) containing a clear watery sap which can be extracted with little or no contamination. In both these the concentration of iodide in solution in the sap greatly exceeds that in the sea water.

In both cases the chemical potential of  $\text{NaI}$ ,  $\text{KI}$ ,  $\text{HI}$ , and  $\text{CaI}_2$  is greater inside than outside. It would therefore seem that an expenditure of energy on the part of the cell is necessary to bring this about.

It is well known that the halide concentration of the sap of both *Valonia macrophylla*, Kütz., and *Halicystis Osterhoutii*, Blinks and Blinks, is greater than that of the sea water. In Bermuda sea water the total halide concentration is about 0.58 M, in the sap it is usually more than 0.60 M but seldom as much as 0.64 M. This accumulation of total halide amounts to 10 per cent at the most. The object of the present paper is to show that iodide accumulates to a greater extent, particularly in the case of *Halicystis*.

In the course of work on the rate of entrance of iodide into *Valonia* and into *Halicystis* the sap from untreated cells was analyzed, and it was found that *Valonia* contained no iodide detectable by the analytical procedure used at the time, but there was invariably an appreciable concentration in *Halicystis* sap. Subsequently a more sensitive method was used for *Valonia* sap which revealed that there is some accumulation in it also.

TABLE I  
*Analyses of Sap of Halicystis Osterhouti for Iodide*

Description of the cells	Treatment prior to sap extraction	Concentration of iodide
		M
1 Attached cells from several sources	Kept in the laboratory in running sea water up to 12 mos	0 000484
2 Attached cells from Tuckers Town 0.5 to 1.5 ml in volume	Sap extracted immediately after collection	0 000407
3 " " " "	" " " " "	0 000295
4 " " " "	Kept in laboratory in running water 4 hrs	0 000253
5 Attached cells from Tuckers Town 30 small cells 0.5 ml or less	Kept in laboratory 3 wks in running sea water	0 000655 0 000642
		Av 0 000648
6 Attached cells from several sources	Kept in laboratory in running sea water 12 to 18 mos	0 000452
7 Attached cells from Tuckers Town 4 large cells 1 ml or more	" " " " "	0 000335
8 Unattached large pale cells stranded on beach 1.5 ml or more in volume	Kept 24 hrs in laboratory in running sea water	0 000256
9 One large unattached cell floating at sea off Coopers Island 3 ml	Sap extracted immediately after collection	0 000204
10 3 large unattached cells stranded on beach at Ft St Catherine 2 to 4 ml in volume	Kept in laboratory in running sea water 4 days	0 000387
11 Unattached cells floating in Ferry Reach 0.75 to 2.5 ml in volume	Kept in laboratory in running sea water	0 000453
12 Unattached cells floating at sea 1/4 mile west of Gurnet Rock, less than 1 ml in volume	Kept in laboratory in running sea water 24 hrs	0 000279
13 Unattached large cells, 2 to 4 ml in volume	" " " " "	0 000289

### Analysis

The method of analysis usually used for *Halicystis* was that described in a previous paper,<sup>1</sup> in which the iodide was oxidized to free iodine by potassium iodate

<sup>1</sup> Jacques, A. G., *J. Gen. Physiol.*, 1936-37, 20, 737

in the presence of tartaric acid. But in some cases  $\text{KMnO}_4$  was used to oxidize the iodide. Results agreeing within the limits of error of the method were obtained on the same sap samples. Before analysis the sap was centrifugalized to remove insoluble organic matter.

For *Valonia*, a 2 ml sample of centrifugalized sap was first treated with 0.5 ml of saturated bromine water and 0.2 ml of 5 per cent acetic acid, whereby the iodide present was oxidized to iodate. Then the excess bromine was removed by boiling and an excess of KI was added. The iodine formed was extracted into chloroform and determined photometrically with the Zeiss Pulfrich step photometer.

The sea water was analyzed for total inorganic iodine by the method of Reith and for iodide ion by the method of Winkler.<sup>2</sup>

The analyses were made in Bermuda at various times in the winter of 1935-36, and of 1936-37 on the sap of *Halicystis* cells from various sources. Some of the cells were found growing attached, while others were stranded on beaches or floating at sea.

The *Valonia* sap analyzed was taken from cells of a composite collection made at various times in the winter of 1936-37.

Table I gives the results for *Halicystis* which was most extensively investigated.

The concentration of iodide in *Valonia* sap was found to be  $1.1 \times 10^{-5} \text{M}$ .

In the sample of Bermuda sea water analyzed the iodide was found to be 5  $\gamma$  per liter and total inorganic iodine 30  $\gamma$  per liter. This is a little lower than Reith<sup>2</sup> has found for total inorganic iodine, his highest value being 69.5  $\gamma$  for the very concentrated Red Sea, and his lowest, 43.4  $\gamma$ , for the North Sea. His values are all somewhat higher than those obtained by other recent investigators. Several older determinations give much higher values, but these are undoubtedly unreliable owing to analytical errors. On the whole, therefore, we believe our value of 30  $\gamma$  per liter to be a sufficiently reliable one.

#### DISCUSSION OF RESULTS

The average concentration of iodide for attached cells of *Halicystis* (1 to 7, Table I) is 0.000410 M, and for floating and stranded cells

<sup>2</sup> Reith J. F. *Rec trav chim Pays bas* 1930 49, 142.

<sup>3</sup> Winkler L. W. *Z angew Chem* 1916 29, 205.

(8 to 13, Table I) 0 000311 M But this difference is probably not significant Greater differences occurred among the members of each group The average for the whole series is 0 000361 M

The accumulation of iodine by some of the algae has, of course, been known for a long time, and several qualitative and quantitative analyses have been made<sup>4</sup> Most of these investigations deal with the brown algae which are the richest in iodine (especially *Laminaria*) and in most cases the results are expressed in terms of per cent of fresh or dry weight, without reference to the form in which iodine occurs or to its concentration

Kylin,<sup>4</sup> however, believes that iodine occurs in easily decomposed organic combinations and as iodides, but never as free iodine in normal plants, which is also the view of Chemin and Legendre,<sup>4</sup> and Mangelot But Dangeard<sup>4</sup> and Sauvageau<sup>4</sup> believe that free iodine is a normal constituent of some cells Dillon<sup>5</sup> suggests that iodine is stored by the union of free iodine in the plant with unsaturated organic compounds, while Freundler<sup>4</sup> and his co-workers have stated that in *Laminaria* (which they have investigated thoroughly) at certain times of the year up to one half the total iodine is present in a form which cannot be demonstrated analytically even though the plant is ashed Freundler<sup>6</sup> has suggested that part of the iodine might at certain times be present as "latent iodine" which he regards as an isomer of iodine and a higher isotope of tin of atomic weight 127 and atomic number 50 This form is supposed to be transformable to ordinary iodine under certain conditions Freundler<sup>7</sup> and his co-workers have also suggested that in the case of *Laminaria* which, according to their analyses, contains tin and rubidium, complexes involving sodium, tin, and iodine, and rubidium, tin, and iodine, are concerned in the latent iodine problem

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<sup>4</sup> Okuda, Y, and Eto, T, *J Coll Agric*, Tokyo, 1916, 5, 341 Freundler, P, Laurent, Y, and Menager, Y, *Bull Soc chim France*, 1922, 31, series 4, 1341 Freundler, P, *Compt rend Acad sc*, 1924, 179, 1421 Lelievre, J, and Menager, Y, *Compt rend Acad sc*, 1924, 178, 1315 Freundler, P, Laurent, Y, Menager, Y, and Lelievre, J, *Bull Soc chim France*, 1925, 37, series 4, 1466 Chemin, E, and Legendre, R, *Compt rend Acad sc*, 1926, 183, 904 Sauvageau, C, *Rev bot appl Agr Coll*, 1926, 6, 169 Chemin, E, *Bull Soc bot France*, 1928, 75, 540 Mangelot, G, *Compt rend Acad sc*, 1928, 186, 93 *Bull Soc bot France*, 1928, 75, 519 Dangeard, P-A, *Bull Soc bot France*, 1928, 75, 509 Kylin, H, *Z physiol Chem*, 1929, 186, 50 Dangeard, P-A, *Compt rend Acad sc*, 1929, 189, 862 Lunde, G, and Closs, K, *Biochem Z*, Berlin, 1930, 219, 198 Trofimov, A, *Planta*, 1934, 23, 56

<sup>5</sup> Dillon, T, *Nature*, 1929, 123, 161

<sup>6</sup> Freundler, P, *Introduction a l'étude des complexes biologiques*, Paris, 1928, p 208

<sup>7</sup> Freundler, P, and Laurent, Y, *Compt rend Acad sc*, 1924, 179, 1049, *Bull Soc chim France*, 1925, 37, series 4, 1133

These views have not met with much approval, most investigators believing that faulty analytical procedures are responsible for the curious results obtained.

Kylin<sup>4</sup> believes that most of the iodine in *Laminaria* is in the form of iodide. But Lunde and Closs<sup>4</sup> found that for different parts of the plant the percentage of iodine in the form of iodide varies from 89 to 36 per cent.

Only one investigator, Trofimov,<sup>4</sup> has attempted to determine the actual concentrations of iodide in tissues of algae. His potentiometric method, involving a silver needle electrode, which is thrust into the tissue, is open to large errors, but gives approximate values for the activity of the iodide ion. But the needle traverses many cells and intercellular spaces, so that the potential observed is not due to cell constituents alone.

His results for *Laminaria* indicate iodide concentrations between 0.00004 M and 0.0030 M, depending on the species and on the kind of tissue. He states that in every tissue a considerable part of the iodine present was not in the form of iodide.

According to this view higher and lower iodide concentrations than those we have found for *Halicystis* appear to exist in *Laminaria* tissues. But owing to the complex structure of the plant and the smallness of the cells it is difficult to deal with it as a physicochemical system, since we have no way of knowing the iodide concentration in the sap of a single cell or of its environment.

In *Halicystis* and *Valonia* this difficulty disappears. There seems no reason to doubt that all of the iodine in the sap of *Halicystis* is in the form of iodide ion. This is indicated by the ease with which it was oxidized to free iodine by potassium iodate and even by nitrite in the presence of a weak acid, viz, tartaric. The case for *Valonia* is not quite so clear. In order to demonstrate the presence of iodine it was necessary to oxidize energetically with bromine water, so that organic compounds of iodine if present would also be included in this analysis. The possibility that the iodine is in the form of an easily decomposable iodo compound cannot be excluded completely. But this seems unlikely in view of the small amount of organic matter in the sap (1.4 parts per 1000).<sup>8</sup>

If all the iodine in Bermuda sea water is taken as iodide the concentration is  $2.4 \times 10^{-7}$  M, or if the iodide is taken as 5  $\gamma$  per liter as

<sup>8</sup> Cf. Osterhout W J V, *J Gen Physiol* 1922-23, 5, 225

we have found, iodide =  $4 \times 10^{-8}M$  But it is generally believed that the greater part of it is present as iodate Thus Winkler<sup>3</sup> estimated that in the Adriatic Sea the iodide content was 11  $\gamma$  per liter and the iodate content 40  $\gamma$  And Cameron<sup>9</sup> found for the Straits of Georgia, British Columbia, 2.5  $\gamma$  iodide and 22.5  $\gamma$  iodate

Assuming that all the iodine in the sap is iodide, and taking our value for the iodide of the sea water, it appears that the iodide has been accumulated by *Halocystis* nearly 10,000-fold Such a change even though the quantities involved are small may indicate a considerable energy change Even if we take all the iodine of the sea water as iodide the accumulation is still more than 1000-fold

In *Valonia*, the accumulation is either 250-fold or 40-fold on this basis Experiments on the penetration of iodide into *Valonia*<sup>1</sup> suggest that the iodide may pass through the protoplasm as sodium iodide But in *Valonia* the concentration of sodium in the sea water is only about 5 times that of the sap and this is not enough to raise the concentration product  $[Na][I]$  in the sea water to that in the sap<sup>10</sup> There is therefore no energy of diffusion available here for the accumulation of iodide On the contrary the movement should be outward rather than inward

The same argument, with greater force, applies to *Halocystis*, where the internal and external concentrations of sodium are about equal What is here said of NaI applies to KI and HI But in *Valonia* the chemical potential of  $MgI_2$  is possibly greater in the sea water than in the sap<sup>11</sup> Hence it might be suggested that  $MgI_2$  enters faster than NaI and KI come out, but this seems highly improbable

It might be assumed that the iodine goes into the cell as iodate and is there reduced to iodide, which might be unable to pass through

<sup>9</sup> Cameron, A. T., *Contrib. Canad. Biol.*, 1922-24, 1, 73

<sup>10</sup> Assuming that the mean activity coefficients for NaI inside and outside are about equal

<sup>11</sup> Our analyses give a trace of magnesium in *Valonia* sap, but Steward and Martin (Steward, F. C., and Martin, J. C., *Carnegie Institution of Washington, Pub. No. 475*, 1937, 87) found in Tortugas the average magnesium concentration to be 0.0036  $M$  in which case, on the basis of 0.057  $M$  magnesium in the sea water, the gradient of chemical potential of  $MgI_2$  is also directed outward

the protoplasm. But in *Valonia* iodide appears to penetrate the protoplasm.<sup>1</sup>

It might be suggested that in *Valonia* the iodine is accumulated in an undissociated organic combination in the sap. If this compound were to leave the cell much more slowly than iodide entered, accumulation might be observed.

It is certain that the cell has the energy available to bring about the effects observed, but the mode of application of this energy is not clear.

While the occurrence of iodine in the green algae is rare it is of interest that some of the algae related to *Valonia* and *Halicystis*, viz *Cladophora rupestris*, *Acrosiphonia pallida*, *Bryopsis plumosa*, and *Bryopsis hypnoides*, are also stated to have iodine. In the case of the latter, according to Dangeard<sup>12</sup> the quantity is about the same as in *Laminaria*.

#### SUMMARY

Analyses of the sap of *Halicystis Osterhoutii* and of *Valonia macrophysa* for iodide indicate accumulations of the order of 1000 to 10,000 fold in the first case, and 40 to 250-fold in the second case. The chemical potential of KI, NaI, HI, and  $\text{CaI}_2$  is greater inside than outside.

<sup>12</sup> Dangeard P. A., *Compt rend Acad sc*, 1929, 189, 862





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#### CORRECTION

In Vol. 21 No. 5, May 20 1938, page 631, in the last line of the first paragraph for " $\text{Na}^+ (U)$ " read " $\text{Na}^+ (U_{\text{Na}})$ "



# SUCROSE INVERSION BY BAKERS' YEAST AS A FUNCTION OF TEMPERATURE

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(Accepted for publication, February 5, 1938)

The velocity of many physiological processes increases exponentially with temperature according to the Arrhenius equation

$$k = Ae^{-\mu/RT}$$

where  $k$  is the rate of the reaction,  $a$  is a constant,  $e$  is 2.718,  $R$  is the gas constant,  $T$  is the absolute temperature, and  $\mu$  represents the energy of activation in calories per mol of the pacemaker reaction in the catenary series of events controlling the rate (Crozier and Hoagland, 1934). Since most biological reactions are catalyzed by enzymes, the change in rate is determined by the enzyme, just as in chemical systems the temperature characteristic is indicative of the specific catalyst employed in the reaction. For certain isolated enzyme systems the data on rate of reaction as a function of temperature fit the Arrhenius equation. For yeast invertase, using different experimental conditions and measuring rate of inversion in a variety of ways,  $\mu$  was found to be independent of temperature and has the value of 11,000–11,500 (Sizer, 1937, 1938). Crozier reported (1924) a constant  $\mu = 16,700$  obtained by analyzing Quastel's data on succinic dehydrogenase of *E. coli*. Craig (1936) isolated a fat oxidation system from *Lupinus albus* and obtained a  $\mu = 11,700$ , when the system was saturated with oil, but the situation was more complicated if oil were not added. Certain other enzyme systems yield constant temperature characteristics at least over narrow ranges of temperature (Barnes, 1937).

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The ultimate interpretation of temperature characteristics for physiological processes will depend on the isolation and study of the chemical reactions involved, especially the slowest in the series and those capable of becoming the pacemaker under different environmental conditions. One approach to the problem is to compare, as a function of temperature, the kinetics of a chemical process carried out inside the living cell with the same reaction catalyzed by an enzyme from that cell. The same temperature characteristic might be expected in the two cases, unless more than one enzyme capable of activating the substrate were present in the cell. In the latter case the temperature analysis might be complex and influenced by unknown factors which determine which one of the several enzymes will play the dominant rôle. A comparison between intracellular behavior of an enzyme and its characteristics after separation from the cell and purification would also be obtained from such a study.

In this study the mechanism of sucrose inversion *in vivo* was measured and compared with enzyme hydrolysis. The kinetics of inversion catalyzed by yeast invertase as a function of temperature was compared with that for inversion using the yeast cells.

#### EXPERIMENTAL

The hydrolysis of sucrose was followed by measuring chemically the amount of reducing sugars present in the digest at successive time intervals. This method has an advantage over the polarimetric technique of following the reaction, since in the former mutarotation of the products of hydrolysis is not a complicating factor. Sumner's dinitrosalicylic acid method (1925, 1935) of measuring colorimetrically the amount of reducing sugars was used, since it had proved to be an excellent method of determining the activity of yeast invertase (Sizer, 1938).

Stock solutions of sugar and yeast were kept in the refrigerator. The sugar solution contained 6 per cent sucrose made up in  $M/15$   $KH_2PO_4$ , and the yeast solution was prepared by making up a 2.5 per cent suspension of compressed yeast in  $M/15$   $KH_2PO_4$ . In the first two series of experiments Fleischmann's bakers' yeast cake was used, while in the last series a pure strain of bakers' yeast, *Saccharomyces cerevisiae* (G M No. 21062 supplied by the Fleischmann laboratory), was employed. This strain is essentially the same as the yeast in yeast cake and was used by Stier and Stannard (1937) in their quantitative study of carbohydrate metabolism. Both stock solutions were adjusted to the temperature of the water bath (temperature controlled to  $\pm 0.05^\circ C$ ) and then 2 cc. 2.5 per cent yeast suspension were added to 10 cc. 6 per cent sucrose. The yeast suspension was rendered homogeneous by thoroughly shaking before using. At successive time

intervals, 1 cc samples of the digest (stirred before removing sample) were added to 3 cc dinitrosalicylic acid reagent in 25 cc Folin Wu sugar tubes. The tubes were kept in a boiling water bath for 5 minutes, then placed under the tap for 3 minutes, and finally diluted to 25 cc. The amount of reducing sugars present was determined by comparing these solutions in a colorimeter with a standard solution treated in a fashion similar to that used on the samples of the digest. A standard containing 5 mg glucose/cc was used in all the work with bakers' yeast but a 4 mg standard was used for work with the pure strain of yeast. Results were expressed in terms of milligrams of reducing sugar per cubic centimeter of digest.

### *Sucrose Hydrolysis by Bakers' Yeast Cake*

Sugar inversion catalyzed by Fleischmann's yeast cake was studied as a function of digestion time and of temperature. When the concentration of reducing sugars is plotted as a function of the duration of digestion, the points fall along a smooth curve (Fig 1, upper curve). This curve is identical in appearance with that obtained using yeast invertase as the catalyst (Sizer, 1938). Rate of digestion can be calculated from the curve by determining the reciprocal of the time required to produce a given amount of invert sugar. Rate expressed as milligrams of invert sugar per minute was calculated from the time required to produce 5 mg invert sugar as indicated by the curve drawn through the plotted points. Rate can be calculated equally as well from other phases of the reaction, although, during the later stages of inversion, interfering factors such as enzyme destruction and inhibition by the accumulated glucose and fructose may complicate the situation.

When log rate is plotted against  $1/T$  (Fig 2, upper curve), the data are best fitted by drawing through the points two straight lines which intersect at a critical temperature of  $17^{\circ}\text{C}$ . The large number of experimental points obtained indicate that the data can be fitted only by drawing through them two straight lines. The slope of the line drawn through the points for from 0 to  $17^{\circ}\text{C}$  corresponds to a  $\mu = 10,700$ . This agrees closely with the value of 11,000 which Sizer (1938) reported over the same temperature range using a similar technique with yeast invertase. The data of Euler and Laurin (1920) indicate a  $\mu = 10,700$  between 0 and  $20^{\circ}\text{C}$  for yeast invertase, although they agree with other workers that  $\mu$  decreases with rise in temperature. This agreement between the  $\mu$  values for yeast and yeast

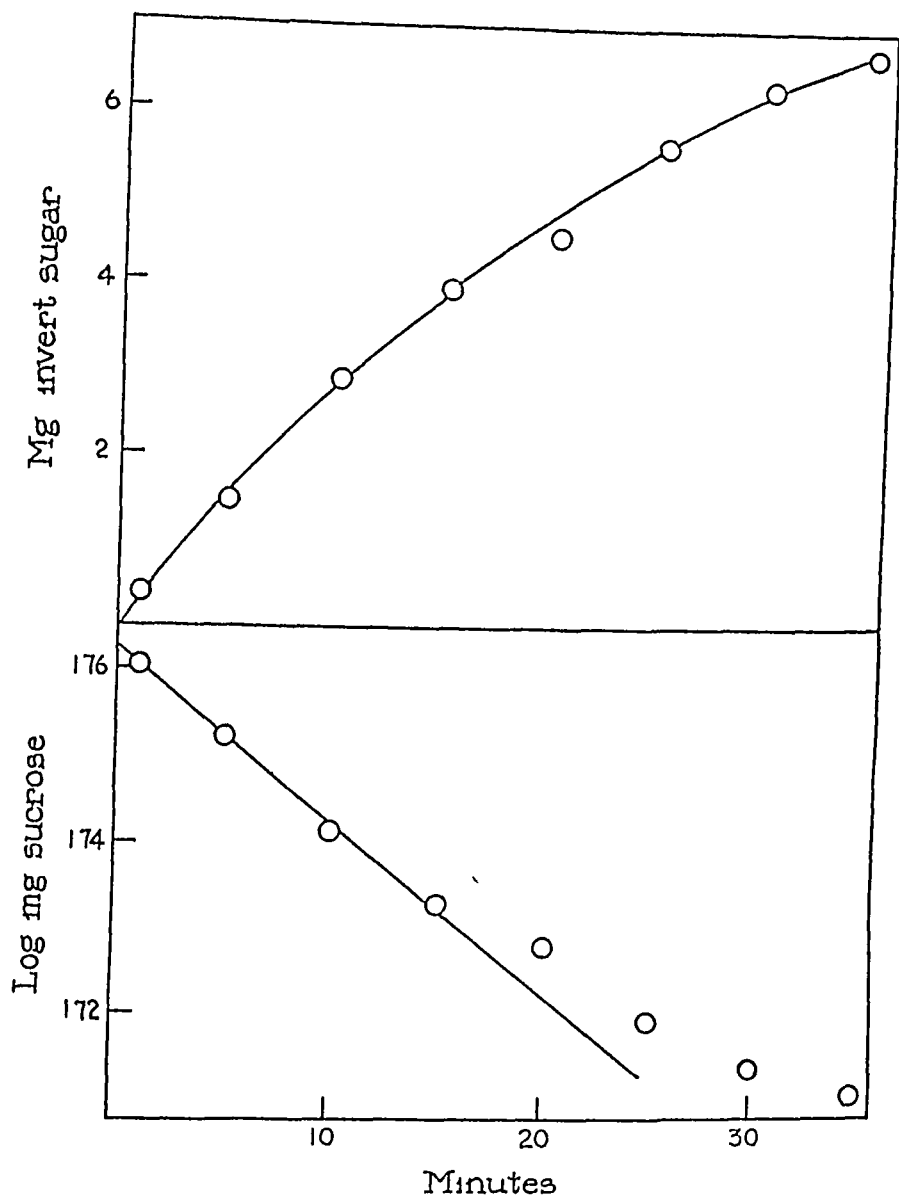


FIG 1 Inversion of 5 per cent sucrose catalyzed by 0.417 per cent bakers' yeast at 21°C

*Upper Curve*—Milligrams invert sugar liberated during the hydrolysis plotted against elapsed time in minutes

*Lower Curve*—Log sucrose concentration ( $\log (58 - \text{milligrams invert sugar})$ ) plotted against the duration of digestion in minutes Monomolecular velocity constant = 0.00200



invertase is evidence that the enzyme behaves identically *in vivo* and *in vitro*, at least with respect to temperature activation. Above  $17^{\circ}\text{C}$  the straight line drawn through the plotted points has a slope corresponding to a  $\mu = 8,300$ . Above  $40^{\circ}\text{C}$  the points fall off from the curve indicating temperature inactivation of the enzyme system.

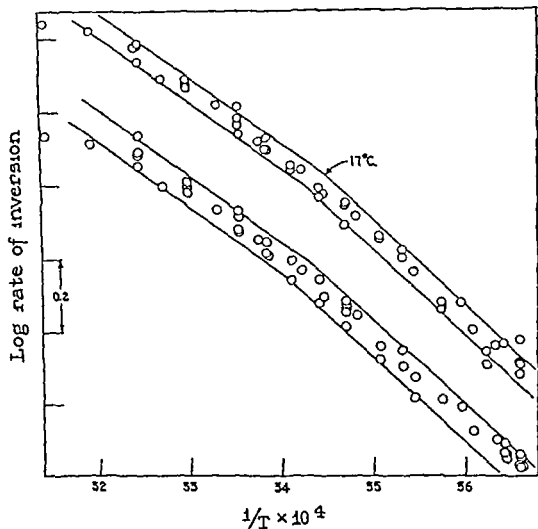


FIG 2 Log rate of inversion of 5 per cent sucrose by 0.417 per cent bakers' yeast plotted against the reciprocal of absolute temperature. Below  $17^{\circ}\text{C}$   $\mu = 10,700$ ; above  $17^{\circ}\text{C}$   $\mu = 8,300$ . Inactivation occurs above  $40^{\circ}\text{C}$ .

Upper Curve—Rate expressed as milligrams invert sugar/min

Lower Curve—Rate expressed as monomolecular velocity constants

The new  $\mu$  value of 8,300 suggests that in the living yeast there exists another enzyme (e.g. yeast maltase) capable of hydrolyzing sucrose, and which plays the dominant rôle in inversion above  $17^{\circ}\text{C}$ . Yeast invertase, on the other hand, is the important catalyst for sugar inversion below that temperature.

Other methods are available for calculating rate of inversion. For example, the monomolecular velocity constants may be computed from a monomolecular plot of the data and used as a measure of rate. When log sucrose concentration is plotted against elapsed time (Fig 1, lower curve), the first few points fit roughly along a straight line, *i.e.*, the reaction follows the monomolecular equation. After about 4 mg reducing sugar have been liberated the points fall off from the straight line. This phenomenon is precisely that obtained with yeast invertase (Sizer, 1938). Monomolecular velocity constants were calculated from the slopes of the straight lines drawn through the plotted points for the various temperatures. The graph of  $\log k$  vs  $1/T$  (Fig 2, lower curve) is practically identical with that obtained when log rate, expressed as milligrams invert sugar/minute, is related to  $1/T$ . It is obvious that results obtained on the kinetics of inversion by living yeast as a function of temperature are independent of the method of analyzing the experimental data. It should be emphasized, however, that both methods employed deal only with data for the first part of the hydrolysis. It seems likely that information obtained for later stages of hydrolysis could not be interpreted in terms of the Arrhenius equation due to interfering factors which complicate matters. A similar situation was postulated for yeast invertase.

#### *Sucrose Inversion by Yeast Killed with Toluene*

Are the kinetics of inversion as a function of temperature related in any way to vital processes in the yeast, or determined by the morphology of the living cell? A study of inversion catalyzed by dead cells was made to answer this question. Formaldehyde and *o*-cresol were unsatisfactory lethal agents for they not only destroyed the cells, but also almost completely inhibited the enzymic hydrolysis of sucrose. Toluene proved satisfactory for it killed the cells, yet did not inhibit their inversion powers. As would be expected, toluene also does not impede the action of yeast invertase. Although the solution saturated with toluene was lethal, the cells were not cytolyzed, but remained intact, during the 2 weeks that the preparation was used. The sucrases were present and active inside the intact dead cells rather than in the surrounding medium (see p 704).

The inversion curve obtained with the dead cells was very similar

to that procured with the living ones. Rate was calculated from the time required to liberate 5 mg invert sugar, and an Arrhenius plot was made of the data (Fig 3, upper curve). Two straight lines intersecting at 17°C best fit the plotted points. The lower and steeper

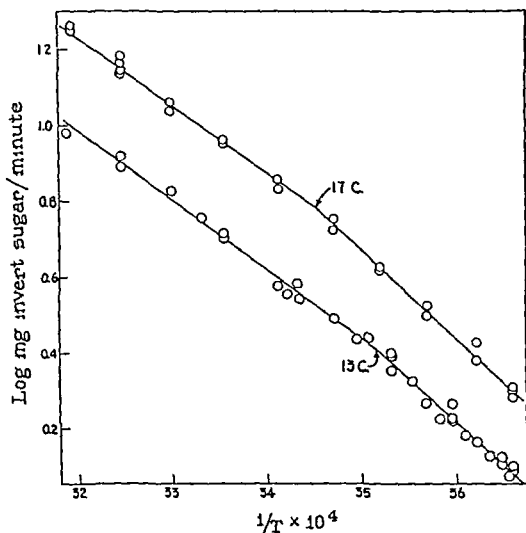


FIG 3 Log 10 times the rate of inversion of 5 per cent sucrose by 0.417 per cent yeast plotted against  $1/T$ . Below 13–17°C  $\mu = 10,700$ ; above this temperature  $\mu = 8,300$ . Inactivation occurs above 40°C.

*Upper Curve*—Inversion catalyzed by bakers' yeast killed with toluene. The critical temperature is 17°C.

*Lower Curve*—Inversion catalyzed by a pure strain of *Saccharomyces cerevisiae* G M No 21062. The critical temperature is 13°C.

line has a slope corresponding to a  $\mu = 10,700$ , while for the upper one  $\mu = 8,300$ . Heat inactivation sets in above 40°C. A comparison of these results on dead yeast with those obtained on living yeast reveals an excellent agreement. It seems evident that the inversion mecha-

nism of the cell is not dependent on vital processes, nor is it determined by that part of the cell structure which changes when death sets in

### *Inversion by a Pure Strain of Yeast*

The common yeast cake may contain more than one strain of yeast as well as certain impurities such as starch which might complicate the inversion picture. In view of this, it was considered advisable to repeat the work on inversion as a function of temperature using a pure strain of *Saccharomyces cerevisiae*. A pure strain of Fleischmann's bakers' yeast, G M No 21062, proved satisfactory for this study.

The course of inversion as a function of the duration of digestion is very similar to that for bakers' yeast cake. Since inversion did not proceed quite as rapidly, however, the rate was calculated from the time required to produce 4 mg invert sugar instead of 5 mg. As before, the time required to produce a given amount of the hexoses was interpolated from the graph. Duplicate determinations were made at each temperature and the plotted points in the Arrhenius graph (Fig 3, lower curve) represent the average of two inversion rates. The analysis of the data for the pure strain yields  $\mu$ 's of 10,700 below 13°C, and 8,300 above that temperature. Enzyme inactivation occurs above 40°C. These results are almost identical with those obtained with bakers' yeast cake, and with yeast killed with toluene. It becomes apparent that the kinetics of inversion as a function of temperature are the same using yeast from different sources and under widely different experimental conditions. It is interesting to point out that the critical temperature for the pure strain is 13°C as compared with 17°C for bakers' yeast.

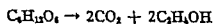
### *Factors Which Might Complicate Inversion*

1 *Change in Number of Living Cells*—An increase or decrease in the number of cells in the stock solution or even a change in their metabolism might alter the kinetics of inversion, especially since the culture was used over a period of several weeks. Yeast cells suspended in M/15  $\text{KH}_2\text{PO}_4$  are so called "resting cells" and do not multiply. Microscopic examination reveals a very small but constant number of budding cells. Neither do they die over the period that they are used, since there is no increase in the number of cells (roughly 5 per cent) which take up the dye when the differential methylene blue

staining technique (Nelson, Palmer, and Wilkes, 1932) was used. This was true for both the ordinary yeast and the pure strain, and agrees with the results of Stier and Stannard (1937). Another indication that no significant change has occurred in the culture is the fact that the rate of inversion at a given temperature does not vary with the age of the yeast suspension. During the course of inversion the sugar solution does not appear to be toxic, since there is no increase in the number of dead cells over a period of several hours. In the case of the yeast treated with toluene about 98 per cent of the cells stained blue indicating that they were dead.

2 *Change in pH of the Digest*—As a result of the normal respiration of the yeast, acids such as carbonic are formed which might inhibit hydrolysis by bringing the sucrases further from the pH for optimum activity (4.7 is the optimum pH for yeast invertase). The increase in acidity of the digest was followed continuously with the glass electrode using a Beckman pH meter. In one experiment the pH decreased from 4.22 to 4.04 in 45 minutes at 24°C. In a second the change was from 4.23 to 3.90 in 83 minutes. A similar increase in acidity was recorded with the quinhydrone electrode in a third test. An average of the three experiments indicated a decrease in pH of about 0.02 unit in the 5 minutes required to liberate 5 mg invert sugar. This slight increase in acidity would not be expected to alter the rate of inversion appreciably.

3 *Fermentation*—As soon as the sucrose is hydrolyzed a part of the invert sugar may be utilized in the systems of respiration and fermentation. This would seriously complicate the measurement of inversion, since it depends upon the determination of the reducing sugars produced. Fermentation was studied using the sensitive micro method for alcohol determination recommended to the author by Dr. Allison and used successfully on yeast by Stier and Stannard (1937). An average of three different experiments indicated that 0.05 mg alcohol was produced at 35°C in the 5 minutes required to liberate 4 mg invert sugar. On the assumption that fermentation may be represented by the equation



(Harrow and Sherwin, 1935) this 0.05 mg represents 0.10 mg hexose which has been fermented. Since, during the liberation of 4 mg

reducing sugars, only 0.10 mg is fermented, it seems evident that during the early part of inversion fermentation does not play a serious complicating rôle

Another indication that fermentation and respiration are not seriously confusing the inversion picture is the fact that, in the toluene-killed cells, the kinetics of inversion as a function of temperature are identical with those for living cells. Yet in the dead cells the processes of fermentation and respiration have been interrupted

### *Is Inversion Extra- Or Intracellular?*

Workers in general agree that yeast invertase is active inside the cell or attached to its surface and is not present in the solution (Nelson, 1933). Since in this work there appear to be two enzymes involved, it seemed advisable to determine whether they were both intracellular. An old suspension of bakers' yeast was filtered through a Seitz filter, and the hydrolytic activity of the cell-free filtrate was tested. The activity was very slight and amounted to only 2.6 per cent of that of the unfiltered material. A similar experiment was performed with the toluene-treated yeast where the filtrate had 5.7 per cent of the activity of the original suspension. In both cases the enzyme activity is associated with intact cells and cannot be readily separated from them.

### DISCUSSION

Warburg and certain others believe that information concerning cellular processes which can be obtained from studying enzyme systems is definitely limited. They suggest that the intracellular behavior of an enzyme may be quite different from its properties after separation from the cell and subsequent purification. There is no evidence for the validity of this statement with respect to yeast invertase. The kinetics of inversion *in vitro* and *in vivo* are the same (Sizer, 1938). Nelson and associates (1932) found that inversion followed the same equation in the two cases. The identity *in vivo* and *in vitro* of the relationships between rate of inversion and  $(H^+)$  or sucrose concentration led Nelson and associates (1933) and Wilkes and Palmer (1933) to the belief that in the living cell invertase was present in or near the cell membrane. Increase in inversion rate as a function of temperature below 13–17°C is also identical in the yeast

cell and yeast invertase solution This lends support to the hypothesis that the change in rate of physiological processes with temperature is determined by the energy of activation of the enzyme-substrate complex of the pacemaker link in the series of reactions controlling the rate

Above 13-17°C, however, there is a sharp contrast between the behavior of the yeast cell and yeast invertase with respect to inversion as a function of temperature For yeast invertase the temperature characteristic is 11,000, while for bakers' yeast above 13-17°C it is 8,300 This difference in  $\mu$  values suggests that in yeast there is present a second sucrase different from the familiar yeast invertase with respect to the fact that it has a lower energy of activation

#### SUMMARY

Inversion of sucrose by bakers' yeast follows the same course as inversion catalyzed by yeast invertase Rate of inversion increases exponentially with temperature, the temperature characteristic in the Arrhenius equation is 10,700 below 13-17°C, and 8,300 above that temperature Temperature inactivation occurs above 40°C The effects of temperature upon rate of inversion were the same using Fleischmann's yeast cake, the same yeast killed with toluene, and a pure strain (G M No 21062) of bakers' yeast The last differed from the other two only in the fact that its critical temperature was 13°C as compared with 17°C for the others

The catalytic inversion is associated with enzyme activity inside the cell, not in the medium, and is independent of any vital processes inside the cell such as respiration and fermentation Since invertase activity is the same inside the cell as it is after extraction, it appears possible to relate the temperature characteristics for physiological processes to the catalytic chemical systems which determine their rate

At least two enzymes are capable of inverting sucrose in the yeast cell The familiar yeast invertase ( $\mu = 10,700$ ) is active below 13-17°C while a second enzyme ( $\mu = 8,300$ ) plays the dominant rôle above that temperature

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# CHANGES OF APPARENT IONIC MOBILITIES IN PROTOPLASM

## III SOME EFFECTS OF GUAIALCOL ON HALICYSTIS

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*Valonia* shows remarkable changes in the apparent mobility of ions as the result of exposure to guaiacol<sup>1</sup>. These changes differ in several respects from those observed in *Halicystis*.

Guaiacol makes the p.d. of *Valonia* more positive and that of *Halicystis* more negative. In *Valonia* the apparent mobility of  $K^+$  is lessened but this does not happen in *Halicystis*. In some respects both plants agree, e.g. in both the apparent mobility of  $Na^+$  is increased by guaiacol.

The effects of guaiacol (called HG for convenience) in sea water at pH 8.2 might theoretically be due to the undissociated molecule or to the ion (which will be called  $G^-$ ). In order to eliminate the effects of  $G^-$  the pH of the sea water was lowered to 6.4. Since this change of pH affects the p.d. it will be described before taking up the effects of HG.

Fig. 1 shows<sup>2</sup> the effect of lowering the pH of sea water from 8.2 to 6.4. After a short latent period<sup>3</sup> the positive<sup>4</sup> p.d. fell<sup>5</sup> from 68 to

<sup>1</sup> Osterhout W J V, *J Gen Physiol*, 1936-37, 20, 13

<sup>2</sup> The experiments were performed on *Halicystis Osterhoutii* (Blinks, L. R., and Blinks A. H., *Bull Torrey Bot Club*, 1930-31, 57, 389), using the technique described in a former paper (see footnote 1). Regarding the amplifier see Hill S. E. and Osterhout, W J V, *J Gen Physiol*, 1937-38, 21, 541. Temperature 21 to 25°C. (The results obtained with this species are not entirely the same with the Pacific Coast *H. ovalis* (Lyngb.) Aresch (Blinks, L. R., private communication).)

<sup>3</sup> The latent period with change of pH and with HG (see footnote 1) varies a good deal and presumably depends to some extent on the state of the cellulose wall. It may in some cases be lengthened by bacterial jelly covering the cells.

55 mv The average of 22 observations gave a change of  $16.5 \pm 0.68$  mv

On returning the cell to sea water at pH 8.2 the  $P_D$  became normal and there was no sign of injury on this or on the following days

When the cell was not returned to sea water at pH 8.2 the  $P_D$  remained practically constant at the lowered value for at least 15 minutes (longer exposures were not made)

Such exposure (up to 15 minutes) did not affect the apparent mobilities of  $K^+$  or  $Na^+$  This was shown by experiments in which the cell after exposure was placed in sea water in which  $K^+$  was increased or in which the sea water was diluted<sup>6</sup> with an equal volume of an isotonic solution of mannite<sup>7</sup> (as described later in experiments with HG)

It may be added that raising the pH of the sea water from 8.2 to 9.6 had little or no effect on the  $P_D$

Blinks<sup>8</sup> states that lowering the pH from 8.2 to 6.0 has no immediate effect on the  $P_D$  The writer has found in a few instances that a change from 8.2 to 6.4 had little or no effect The cells which show no change in  $P_D$  do not seem to be injured The high positive  $P_D$  which is normal for *Halocystis* in sea water at pH 8.2 is present in these cells and they respond normally to increases in the concentration of KCl or to dilution of the sea water Their behavior depends

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(this may give them a slippery feeling which was not present in the cells here described) A latent period was observed with KCl and with 0.005 M  $NH_4Cl$  at pH 8.2 (but not with 0.3 M  $NH_4Cl$  at pH 5.8, 6.4, and 7.2, the  $P_D$  being rendered negative in each case), and with sea water diluted with an isotonic solution of non-electrolyte In the presence of 0.01 M HG there was no latent period with potassium sea water (see Figs 7 and 8)

<sup>4</sup> The  $P_D$  is called positive when positive current tends to flow from the sap across the protoplasm to the external solution

<sup>5</sup> The average value is 67 mv (cf Blinks, L. R., *J. Gen. Physiol.*, 1933-34, 16, 147)

<sup>6</sup> Cf p 714

<sup>7</sup> Regarding this see Osterhout, W. J. V., *Proc. Nat. Acad. Sci.*, 1938, 24, 75

<sup>8</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 229 Blinks states that lowering the pH to 5 makes the  $P_D$  reversibly less positive (Blinks, L. R., *J. Gen. Physiol.*, 1933-34, 17, 118, Fig 7) In the cells dealt with in the present paper this treatment carried the  $P_D$  reversibly to zero See also Blinks, L. R., *J. Gen. Physiol.*, 1934-35, 18, 409

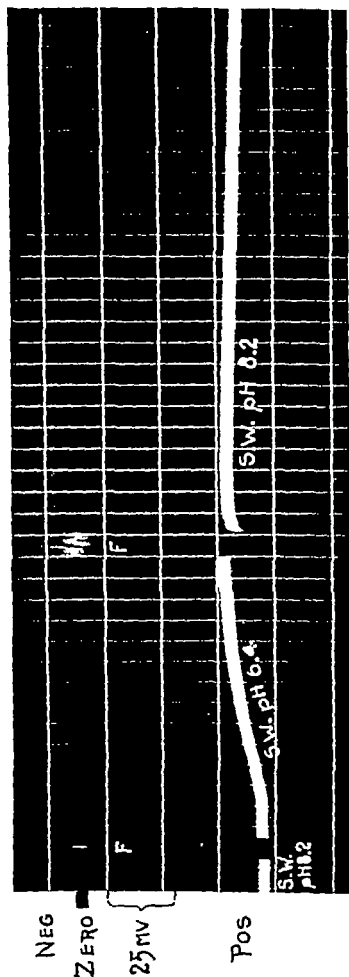


FIG. 1 Photographic record showing change of  $pD$  produced by lowering the  $pH$  of sea water from 8.2 to 6.4. At the start the cell in sea water at  $pH$  8.2 had a positive  $pD$  of 68 mv. When it was lifted out of the sea water the curve jumped to 1 the 'free grid' of the amplifier. When it was placed in sea water at  $pH$  6.4 the curve jumped back to its former position and after a few seconds (latent period) began to rise the  $pD$  becoming less positive to the extent of 13 mv.

The cell was then replaced in sea water at  $pH$  8.2 and the curve returned to the starting point. If the cell is left in sea water at  $pH$  6.4 the  $pD$  remains at the maximum for at least 15 minutes (longer exposures were not made). Time marks 5 seconds apart. Temperature 22°C.

on a factor which is at present unknown. Possibly the cell can exist in two or more physiological states. (Blinks<sup>9</sup> has described for *Valonia* two states known as "regular" and "delayed" in respect to polarization.)

How the lowering of external pH operates in decreasing the  $P D$  is not clear. We may recall in this connection the observation of Blinks<sup>10</sup> that when sufficient ammonia is present and the pH of the sea water is raised above a certain point the  $P D$  suddenly changes from positive to negative.<sup>11</sup> Lowering the external pH then produces positivity, *vice versa*, just the opposite of what happens in the experiments described in this paper. Since the concentration of hydrogen ions is very small in comparison with the other ions present Blinks has suggested that we are here dealing with isoelectric points or similar phenomena.

It is, of course, possible that isoelectric points may be involved in the changes seen in Fig. 1. A variety of ampholytes may be present with different isoelectric points.

Another possibility is that the locus of the changes may not be the same in all cases. The changes shown in Fig. 1 may have their seat in the outer part of the protoplasm. But we must consider the possibility that the pH of the sap may be changed, for the lowering of the external pH would drive  $CO_2$  into the cell.

Let us now consider the effects of guaiacol at pH 6.4 where the concentration of guaiacol ions is negligible.<sup>12</sup> In Fig. 2 we see the record of a cell which had at the start a positive  $P D$  of 59 mv. When the pH was lowered to 6.4 the  $P D$  became 13 mv less positive. When the cell was transferred to sea water at pH 6.4 containing 0.01 M HG the  $P D$  became still less positive to the extent of 23 mv (the average value was  $20.2 \pm 1$  mv in 21 observations). After standing for 1

<sup>9</sup> Blinks, L. R., *J. Gen. Physiol.*, 1935-36, **19**, 633.

<sup>10</sup> Blinks, L. R., *J. Gen. Physiol.*, 1933-34, **17**, 109, 1934-35, **18**, 409.

<sup>11</sup> This does not take place in *Valonia*.

<sup>12</sup> According to Shedlovsky and Uhlig (Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933-34, **17**, 567) the  $pK$  of HG at 25°C is 10.1. Subtracting 0.375 on account of the ionic strength of sea water gives  $pK = 9.725$ . Accordingly at pH 6.4 the degree of dissociation is 0.07 per cent, at pH 8.2 it is 3 per cent, and at pH 9.6 it is 42.9 per cent.

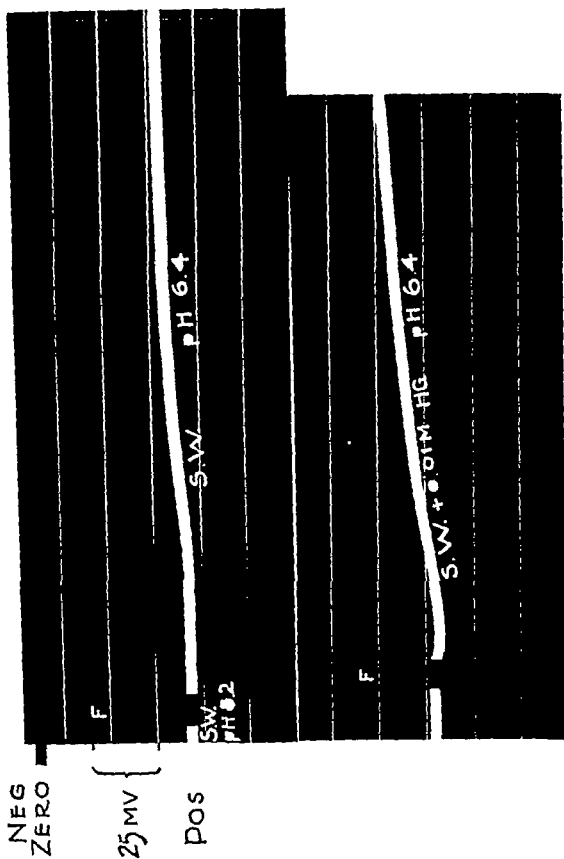


FIG. 2 Photographic record showing changes of  $p.d.$  produced by lowering the  $pH$  and adding sea water. At the start the cell in sea water at  $pH\ 8.2$  had a positive  $p.d.$  of  $59\ mV$ .

The cell was placed in sea water at  $pH\ 6.4$  and after a latent period the curve rose  $13\ mV$  (the  $p.d.$  became less positive). The cell was then transferred to sea water at  $pH\ 6.4$  containing  $0.01\ M\ HG$  and after a latent period the curve rose  $22\ mV$ . When the cell was returned to sea water at  $pH\ 8.2$  the  $p.d.$  became normal (not shown in the figure).

minute the value began to increase and in 5 minutes the  $P D$  was  $40.7 \pm 1.3$  mv (6 observations) less positive than before the application of HG

On standing, even for 15 minutes, there is little or no further change and no recovery of the original  $P D$  such as occurs in *Valonia*<sup>1</sup>

There is quite commonly a slight dip in the curve, soon after the start, as seen in Fig 3 (which shows the effect of adding 0.01 M HG to sea water at pH 8.2) This form of curve is also found at pH 6.4 and intermediate forms between this and the one shown in Fig 2 are

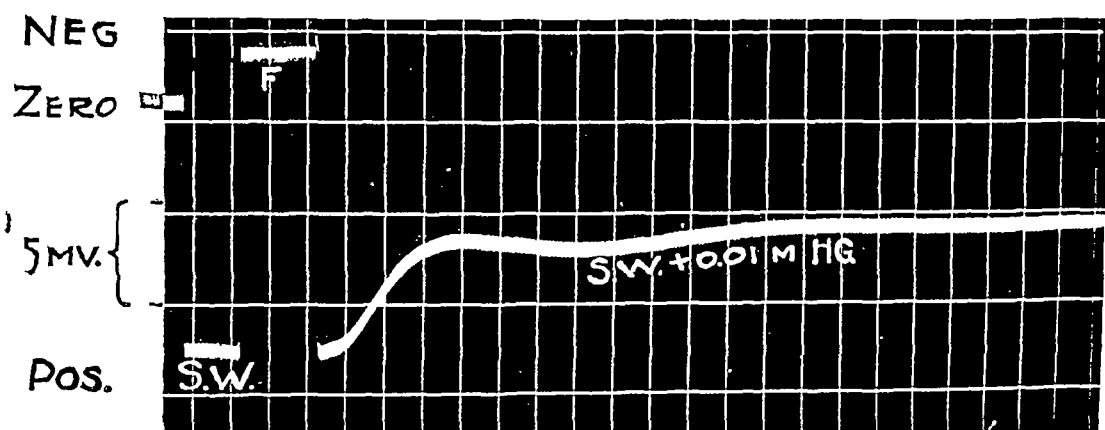


FIG 3 Photographic record showing changes of  $P D$  produced by guaiacol at pH 8.2 At the start the cell in sea water at pH 8.2 had a positive  $P D$  of 62.5 mv

The cell was placed in sea water at pH 8.2 containing 0.01 M guaiacol and the curve rose 30 mv (the  $P D$  became less positive) When the cell was replaced in sea water at pH 8.2 the  $P D$  became normal (not shown in the figure)

Regarding  $F$  see Fig 1

Time marks 5 seconds apart Temperature 21°C

found at both pH values The average change due to 0.01 M HG at pH 8.2 at the end of 1 minute was  $33 \pm 1.3$  mv (17 observations)

Increasing the concentration of guaiacol (at pH 8.2 or 6.4) increases the effect but the value of the  $P D$  appears to approach zero as a limit, i.e. the  $P D$  which in sea water is about 67 mv positive<sup>13</sup> becomes less as the concentration of guaiacol increases but it seldom passes the zero mark so as to become negative (never more than a few millivolts in any case)

<sup>13</sup> Blinks, L. R., *J. Gen. Physiol.*, 1932-33, 16, 147

In the experiments described in this paper the effects were fully reversible. For example, if the  $P.D.$  had been lowered to zero by adding guaiacol it would promptly return to the normal value when replaced in sea water (even after an exposure of 15 minutes to HG). There was no sign of injury in these experiments except with 0.03 M HG or higher concentrations.

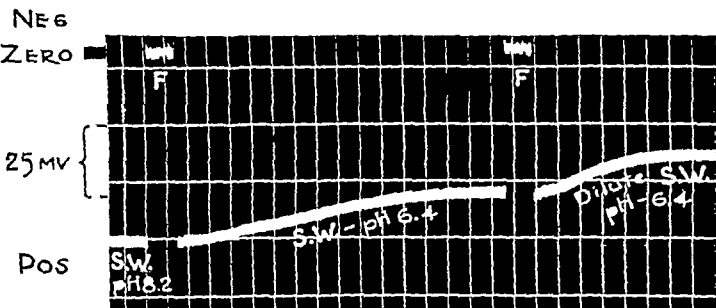


FIG. 4. Photographic record showing changes of  $P.D.$  produced by lowering the pH and diluting the sea water.

At the start the cell in sea water at pH 8.2 had a positive  $P.D.$  of 67.5 mv. When the pH was lowered to 6.4 the curve rose 16 mv (the  $P.D.$  became less positive).

The cell was then placed in dilute sea water (sea water plus an equal volume of a solution containing 1.1 M mannite + 0.02 M CaCl + 0.012 M KCl) and the curve rose 14 mv. When the cell was replaced in sea water at pH 8.2 the  $P.D.$  became normal (not shown in the figure).

Regarding  $F$  see Fig. 1.

Time marks 5 seconds apart. Temperature 21°C.

Let us now consider the effect of the guaiacol ion. In *Valonia* it was found that at first it made little difference whether HG was in molecular form or was largely dissociated. But after standing for about 5 minutes in sea water + 0.01 M HG the cell became sensitized to  $G^-$ . This did not happen with *Halicystis*. Neither at the start nor after exposures up to 15 minutes to sea water (at pH 8.2) contain-

ing 0.01 M HG did a rise of pH from 8.2 to 9.6 (increasing <sup>12</sup> the concentration of  $G^-$  about 14 times)<sup>14</sup> have much effect<sup>15</sup> on the P.D.

There is another aspect of the action of guaiacol which is of considerable interest and which seems to be similar in *Valonia* and in *Halicystis*. After sufficient exposure to HG an application of dilute sea water to *Valonia* made the P.D. more positive (instead of more negative, as happens when the cells have not been exposed to HG)

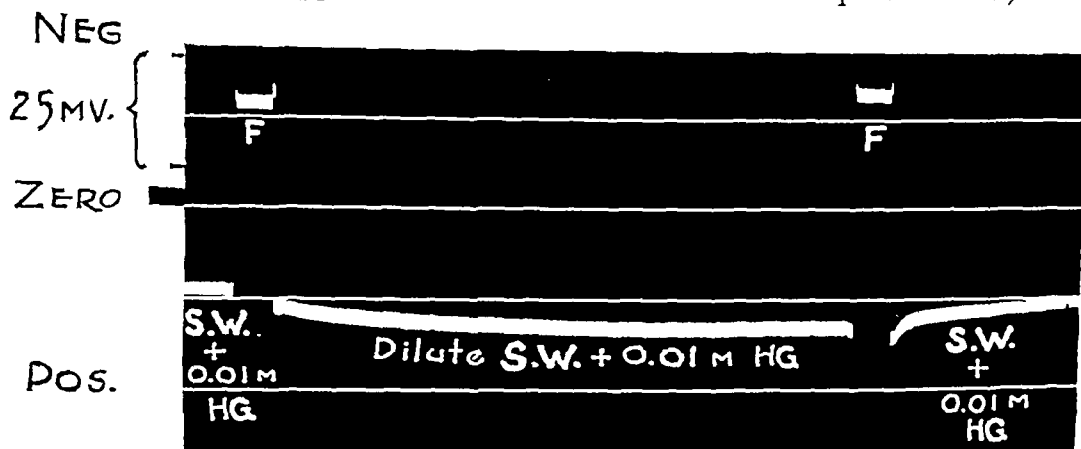


FIG. 5. Photographic record showing changes of P.D. produced by diluting the sea water (all solutions at pH 6.4). At the start of the record the cell had been in sea water containing 0.01 M guaiacol for 15 minutes and had a positive P.D. of 20 mv. (before the addition of guaiacol the P.D. was 65 mv.)

The cell was placed in dilute sea water (sea water containing 0.01 M guaiacol plus an equal volume of a solution containing 1.1 M mannite + 0.02 M  $CaCl_2$  + 0.012 M KCl + 0.01 M guaiacol) and the curve fell 8 mv., i.e., the P.D. became more positive. (In dilute sea water without HG the curve rises and the P.D. becomes more negative; compare Fig. 4.)

Regarding *F* see Fig. 1

Time scale 40 seconds to the inch. Temperature 23°C

This also occurs in *Halicystis*. Fig. 4 shows the normal effect of dilute sea water.<sup>7, 10</sup> Fig. 5 shows the effect after an exposure of 15

<sup>14</sup> It has already been stated that in the absence of HG such a rise has little or no effect. In the presence of 0.01 M HG a rise from pH 6.4 to 8.2 or to 9.6 makes the P.D. more positive to about the same extent as in the absence of HG; hence we may suppose that it is not due to  $G^-$ .

<sup>15</sup> If any change occurred it was at most 3 mv., sometimes in a positive and sometimes in a negative direction.

<sup>16</sup> This value was about the same as for *Valonia* (cf. Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 445).



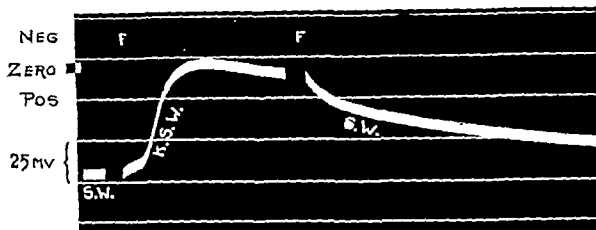


FIG 6 Photographic record showing changes of P.D. produced by potassium (all solutions at pH 8.2). The cell in sea water at the start had a positive P.D. of 65 mv. It was placed in sea water containing  $0.52 \text{ M KCl} + 0.012 \text{ M NaCl}$  (the other constituents of the sea water had the normal concentration) this is labelled K.S.W. in the record. With no latent period the curve rose to 1 mv negative and then began to drop as the P.D. became more positive. When the cell was replaced in sea water the P.D. became normal.

A similar result was obtained at pH 6.4.

Regarding the record at F (invisible in the figure) see Fig. 1

Time marks 5 seconds apart. Temperature  $25^{\circ}\text{C}$ .

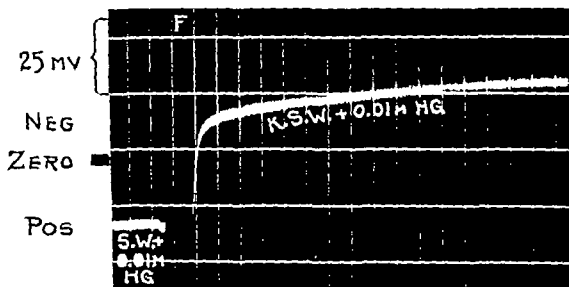


FIG 7 Photographic record showing changes of P.D. produced by potassium after exposure of the cell for 10 minutes to  $0.01 \text{ M}$  guaiacol (all solutions at pH 6.4). (Before exposure to guaiacol the P.D. in sea water at pH 8.2 was 65 mv positive.) At the start of the record the cell in sea water containing  $0.01 \text{ M}$  guaiacol had a positive P.D. of 23 mv. The cell was placed in sea water containing  $0.01 \text{ M HG} + 0.52 \text{ M KCl} + 0.012 \text{ M NaCl}$  (the other constituents of the sea water had the normal concentrations) this is labelled K.S.W. +  $0.01 \text{ M HG}$  on the record. The curve rose (with no latent period) and the P.D. became 23 mv negative.

Dilution of this solution (not shown in the record) with an isotonic solution of mannite (as in Fig. 5) made the P.D. 10 mv positive.

Regarding the record at F (invisible in the figure) see Fig. 1

Time marks 7 seconds apart. Temperature  $23^{\circ}\text{C}$ .

minutes<sup>17</sup> to sea water containing 0.01 M HG at pH 6.4 (the greatest change found in 12 trials was 9 mv<sup>18</sup>)

As in the case of *Valonia* we may<sup>1</sup> interpret this to mean that HG greatly increases the apparent mobility of  $\text{Na}^+$  (as compared with that of  $\text{Cl}^-$ ) in the outer non-aqueous protoplasmic surface. In normal cells (Fig. 4) the apparent mobility of  $\text{Na}^+$  is much less than that of  $\text{Cl}^-$  but in Fig. 5 it is much greater than that of  $\text{Cl}^-$ .

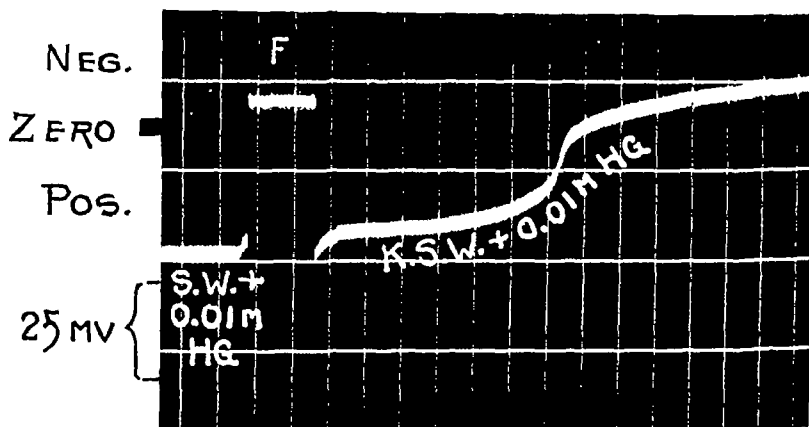


FIG. 8 Photographic record showing changes of P.D. produced by potassium after exposure of the cell to guaiacol (all solutions at pH 8.2). The cell in sea water containing 0.01 M HG had at the start of the record a positive P.D. of 29 mv (originally in sea water it had a positive P.D. of 66 mv). The cell was placed in sea water containing 0.01 M HG + 0.52 M KCl + 0.012 M NaCl (the remaining constituents of sea water had their normal concentrations) this is labelled K.S.W. + 0.01 M HG on the record. The curve, with no latent period, rose to 13 mv negative.

When the cell was replaced in sea water + 0.01 M HG the P.D. returned to the starting point (not shown in the figure).

Regarding *F* see Fig. 1

Time marks 5 seconds apart. Temperature 21°C

In some cases we find after exposures to HG up to 15 minutes that no change occurs when dilute sea water is applied. In such cases we may assume (as in the case of *Valonia*) that the mobility of  $\text{Na}^+$  has become equal to that of  $\text{Cl}^-$ .

<sup>17</sup> This effect may occur after an exposure of only 30 seconds to HG.

<sup>18</sup> This was after an exposure of 11.5 minutes to 0.01 M HG in sea water at pH 6.4.

Another interesting aspect of the action of HG is seen in its effect on the apparent mobility of  $K^+$ . In *Valonia* this mobility is decreased by HG until KCl instead of making the p.d. less positive actually makes it more positive.<sup>1</sup>

*Halocystis* shows no such change in its behavior toward KCl. An application of sea water in which  $Na^+$  has been replaced<sup>10</sup> by  $K^+$  makes the p.d. much less positive whether the cell has been exposed to HG or not. But the form of the curve<sup>20</sup> showing the action of KCl is altered by the exposure to HG, as shown in Figs 6, 7, and 8. We see that the p.d. can become negative under the combined action of KCl and HG.

#### DISCUSSION

Changes of pH have already been considered. The effect of HG remains to be discussed.

In *Valonia*, HG makes the p.d. more positive and this occurs before any change in the apparent mobility of  $K^+$  or  $Na^+$  can be detected. It would therefore seem that the effect of HG concerns their partition coefficients or the organic ions in the cell. It might conceivably change the nature or the concentration of these organic ions or alter their mobilities or partition coefficients in the non aqueous protoplasmic surfaces.

In *Halocystis* the effect of HG on the apparent mobility of  $Na^+$  may occur very early, e.g. after an exposure of 30 seconds or less to HG, which may account, to some extent at least, for the change of p.d. in a negative direction. The apparent mobility of  $Na^+$  is normally less than that of  $Cl^-$  and this tends to make<sup>21</sup> the p.d. more positive. Hence in so far as HG increases the apparent mobility of  $Na^+$  it will make the p.d. less positive.

It would seem, however, that organic ions in the protoplasm may play a part in determining the p.d. Blinks finds that when the solutions at the inner and outer protoplasmic surfaces are identical much of the p.d. persists.<sup>22</sup> This seems to be due to unlike apparent mo-

<sup>10</sup> This contains 0.52 M KCl and 0.012 M NaCl. The other constituents of the sea water remained unchanged.

<sup>20</sup> Osterhout, W. J. V., *J. Gen. Physiol.* 1937-38, 21, 631.

<sup>21</sup> I.e. if it is diffusing inward, as it is in the case of a growing cell.

<sup>22</sup> Blinks, L. R., *J. Gen. Physiol.*, 1934-35, 18, 409.

bilities of the same ions in the inner and outer surfaces. To what extent these are inorganic is not clear but organic ions in the protoplasm and sap may well play a part. Hence it would seem that such organic ions may be involved when  $HG$  lessens the  $P D$ .

In *Valonia* the  $P D$  does not retain the positivity produced by guaiacol but gradually returns to its normal value. This is known as "recovery". It has been interpreted<sup>1</sup> to mean that guaiacol penetrates to the inner protoplasmic surface  $Y$  and there sets up changes which are of opposite sign to those set up in the outer protoplasmic surface,  $X$ . Failure to set up these changes in  $Y$  would prevent recovery and this explanation may apply to *Halocystis*.

If the protoplasmic surface were a pore system in the sense of Michaelis we should be obliged to say that its charge had been changed from negative to positive in *Halocystis* by the action of guaiacol if we are considering  $NaCl$  but not if we are considering  $KCl$ .

If we regard the surface as a non-aqueous layer without pores we may suppose that guaiacol alters mobilities or partition coefficients in this layer. It is quite possible that chemical compounds<sup>23</sup> are formed or complexes in the sense of Kraus and of Fuoss<sup>24</sup>.

We have no explanation at present for the fact that guaiacol changes the sign of the response to  $KCl$  in *Valonia* but not in *Halocystis*.

Another difference between the two organisms is seen when the  $pH$  is lowered from 8.2 to 6.4. This has little or no effect on *Valonia* but usually makes the  $P D$  of *Halocystis* less positive.

In this respect the cells of *Halocystis* are variable and such variability is highly characteristic. In fact it may be said that both plants are variable in many respects. Certain cells fail to show an expected change in  $P D$  in response to external changes such as alterations of  $pH$ , dilution, application of  $KCl$ , or of guaiacol, although evidently not dead, as shown by the  $P D$  in sea water and by the response to certain other external stimuli. It would seem that different physiological states are possible, perhaps analogous to those described by Blinks for *Valonia*.<sup>9</sup>

<sup>23</sup> Guaiacol forms compounds with both acids and bases.

<sup>24</sup> Cf. Kraus, C. A., *Tr. Electrochem. Soc.*, 1934, 66, 179. Fuoss, R. M., *Chem. Rev.*, 1935, 17, 27.

It may be added that although it has been assumed for purposes of discussion that the  $pD$ 's observed are chiefly due to diffusion potential this is done merely because it has been found in previous work<sup>5</sup> that the equations for diffusion potential enable us to predict results with sufficient accuracy. Other explanations may be possible, such as those discussed in a recent paper<sup>26</sup>

The facts brought out in this paper add to the list of striking differences between *Valonia* and *Halocystis*. These differences are of interest as showing the possibilities of protoplasmic systems which were once regarded as very similar.

The fact that organic substances can change the apparent mobilities (as shown by the concentration effect) of inorganic ions (e.g.,  $K^+$  and  $Na^+$  in *Valonia* and  $Na^+$  in *Halocystis*) is of considerable interest. It is shown not only by the action of guaiacol<sup>1</sup> but also by the fact that removal of organic substances from *Nitella* greatly lowers the apparent mobility<sup>27</sup> of  $K^+$  so that the cell ceases to act in the fashion of a potassium electrode. The cell regains this power when treated with guanidine and certain other organic substances<sup>28</sup>

#### SUMMARY

Lowering the pH of sea water from 8.2 to 6.4 lowers the positive  $pD$  of *Halocystis* reversibly (this does not happen with *Valonia*)

Exposure to sea water at pH 6.4 does not affect the apparent mobility of  $Na^+$  or of  $K^+$  (this agrees with *Valonia*)

Guaiacol makes the  $pD$  of *Halocystis* less positive (in *Valonia* it has the opposite effect)

Exposure to guaiacol does not reverse the effect of KCl in *Halocystis* which in this respect differs from *Valonia*

The  $pD$  can be changed from 66 mv positive to 23 mv negative by the combined action of KCl and guaiacol

Exposure to guaiacol affects *Halocystis* and *Valonia* similarly in respect to their behavior with dilute sea water. Normally the dilute

<sup>25</sup> Osterhout W J V, *J Gen Physiol*, 1929-30, 13, 715

<sup>26</sup> Osterhout, W J V *Proc Nat Acad Sci*, 1938, 24, 75

<sup>27</sup> Osterhout, W J V, *J Gen Physiol*, 1934-35, 18, 987

<sup>28</sup> Osterhout, W J V and Hill, S E *Proc Soc Exp Biol and Med*, 1934-35 32 715

sea water makes the P D more negative but after sufficient exposure to guaiacol dilute sea water either produces no change in P D or makes it more positive. In the latter case we may assume that the apparent mobility of  $\text{Na}^+$  has become greater than that of  $\text{Cl}^-$  as the result of the action of guaiacol. (Normally the apparent mobility of  $\text{Cl}^-$  is greater than that of  $\text{Na}^+$ .)

In *Halocystis*, as in *Valonia* and in *Nitella*, an organic substance can greatly change the apparent mobilities of certain inorganic ions ( $\text{K}^+$  or  $\text{Na}^+$ )

## FACTORS LIMITING BACTERIAL GROWTH

### III CELL SIZE AND "PHYSIOLOGIC YOUTH" IN BACTERIUM COLI CULTURES

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It has been repeatedly shown that the metabolic activity in a bacterial culture computed on a rate per cell basis increases during the period of lag to a peak somewhat before the maximum rate of multiplication is attained, and afterward falls off to a very small value. This finding usually has been regarded as evidence that bacteria undergo rejuvenescence prior to active multiplication. Although the underlying observations have been amply confirmed, this interpretation has seemed to us open to question.

The concept of physiologic youth, originally applied to bacteria by Sherman and Albus (1) to signalize their observation that during the period of rapid multiplication bacteria exhibit increased susceptibility to injurious influences, has since become established as the most satisfactory interpretation of the fact that bacteria transferred to sterile broth multiply for a time at a rate which is dependent on the condition of the culture from which they are taken. The observations of Clark and Ruehl (2), and of Henrici (3), concerning the systematic changes in morphology and size of individual cells during the cycle of growth, gave particular credence to this view. Apart from the fact that it is based on doubtful analogy with mammalian physiology, the hypothesis of cellular differentiation has remained of limited usefulness especially because the experiments designed to demonstrate differences in the metabolism of "embryonal," mature, and senescent forms of bacteria, have not been entirely convincing. This has been due to failure to control adequately some variables known to influence the physiology of bacteria in artificial culture.

In some cases the size of individual cells or groups of cells has been left out of account (4), and nearly always the influence of the changing environment itself has been disregarded (4-6). Nevertheless, the observed differences in physiological activity of the cells often have been attributed to differences within the cell itself.

Only a few investigators have realized the necessity of eliminating the effects due to changing external environment. Thus in the original observations of Sherman and Albus (1) on resistance, for example, environmental factors were controlled to the extent that uniform populations were employed. More recently Longsworth and MacInnes (7) were able to maintain a constant pH and the gaseous environment in actively growing cultures of *Lactobacillus acidophilus* and found that under these conditions during the latter part of the growth cycle, while generation time showed a tendency to increase, the fermentation rate per cell was markedly decreasing. Also Moyer (8), who studied cataphoretic mobility of bacteria suspended in buffer solutions, found differences which were related in a consistent manner to the phase of growth of the parent culture. He believed that these differences could not be attributed to variations in the size of the cells. More recently Wooldridge, Knox, and Glass (9) have studied the metabolic activities of non-proliferating bacteria under uniform conditions in relation to the age of the parent culture. These authors found that, among cell suspensions of equal turbidity prepared from cultures of different age, those derived from 24 hour cultures showed maximal activity irrespective of the nature of the substrate. Although the phase of growth of these cultures was not indicated, it is difficult to relate this finding to the frequent observation that heightened activity occurs at the end of the period of lag. When the relation between metabolic activity and viable count was studied by the same authors (10), greater discrepancies were found, but again correlation with phase of growth was not made. In the authors' opinion, these differences could not be attributed to variations in the size of cells.

Clifton (11) has expressed the contrary opinion, for which his experiments have provided only indirect evidence, namely that the metabolic activities of the cells in a bacterial culture are influenced primarily by their size, and by the changing conditions of their



growth Our own preliminary experiments seem to have justified a similar conclusion (12)

The experiments to be described in this paper were designed to measure the physiologic changes which bacteria undergo during the various phases of multiplication, with particular attention to controls suggested by the considerations outlined above The manometric method which was chosen for this purpose was found to yield reliable information as to the rate of oxygen uptake of cells of *Bacterium coli* removed from cultures after various intervals of growth and placed in a uniform medium favorable to continued growth, and to permit the correlation of these data with both the number of viable cells and the total mass of bacterial substance

The bacteria were cultivated in filtered beef extract broth at 37°C in Erlenmeyer flasks filled to a depth of 2 cm, removed from this medium by rapid centrifugation, and suspended without washing in 0.85 per cent saline. It was found that when 100 ml. of the medium were seeded with 0.01 ml of an 18-24 hour culture grown in the same medium, reproducible growth curves were obtained, thus indicating that the phase of growth of cultures of a given age could be predicted The age of the culture was reckoned as the interval between seeding and suspending the centrifuged cells in saline (8) Uniform bacterial content was obtained by suitable dilution of the saline suspension on the basis of nephelometric estimation of bacterial substance. Viable counts were made by the dilution and pour plate method either from the saline suspension or in many experiments, from one of the vessels of the respirometer The population of the original culture was obtained from the viable count of the saline suspension by computing to original volume Oxygen consumption was measured in the Barcroft Warburg apparatus at 37°C, with 3 aliquots estimated from the turbidity of the original suspension to contain respectively the nephelometric equivalent of  $10^8$ ,  $3 \times 10^8$ , and  $10^9$  organisms from a 24 hour culture. The cell suspension was placed in the vessel with sufficient 0.85 per cent saline to bring the total volume to 4 ml, the sidearm containing 1 ml of beef extract medium of such composition that, after mixing concentrations of peptone 1.0 per cent, beef extract 0.5 per cent, and phosphate buffer of pH 6.6 M/50 were obtained The nutrient mixture was added to the bacterial suspension after temperature equilibrium was established (usually requiring 10 minutes) the zero time noted, and the viable count made The readings of the manometers were continued at 5 minute intervals for approximately 1 hour Values for total bacterial substance in the saline suspension were obtained both by means of a photoelectric nephelometer (13) and by determination of bacterial nitrogen by the micro-Kjeldahl method, after a second centri-

fugation to remove soluble nitrogen. In blank determinations, made by adding small numbers of bacteria of known nitrogen content to fresh broth and recentrifuging, no increase in nitrogen was observed.

The average rates of oxygen uptake during the successive 5 minute intervals were spotted at the mid points of corresponding intervals on the graph, and the points connected by a curve smoothed, if necessary, by inspection. Usually the rate for the second, and often for the first 5 minute interval fell on this line. The error introduced by assigning the average rates to the mid points of the 5 minute intervals was too small to be significant. By extrapolation of this curve to the ordinate, the rate of oxygen consumption at zero time, corresponding to the moment at which the substrate was added, was obtained. This value has been used in all calculations.

The rates of oxygen uptake at zero time per milliliter of the original suspension for the three measurements showed satisfactory agreement. Their average divided by the numbers obtained from the viable count, and by the nitrogen content, respectively, gave the rate of oxygen uptake "per viable cell," and "per gram of bacterial nitrogen."

Turbidity was measured by dilution of the suspension to give the zero reading at the resistance box when matched against a 0.5 per cent solution of copper sulfate as standard. The ratio between the average number of viable cells in suspensions from 18-24 hour cultures, after dilution to standard turbidity, and the numbers in the test suspension similarly diluted, expressed the "nephelometric index of size." By dividing the rate of oxygen uptake per cell by this index, the rate "per nephelometric cell" was obtained.

For example, a 3 hour culture is centrifuged and the cells are suspended in 1/20 volume of saline. The following data are obtained

Dilution in saline required to match 0.5 per cent	
CuSO <sub>4</sub> in the nephelometer =	1 8 6
Numbers of 18-24 hr cells giving similar turbidity =	$3.7 \times 10^7/\text{ml}$
-O <sub>2</sub> per hour for 3 1 ml	181.2 mm <sup>3</sup>
0.94 "	54.9 "
0.31 "	17.9 "
1.0 " (average)	58.2 "
Nitrogen content	$1.24 \times 10^{-2} \text{ mg/ml}$
Viable count	$6.0 \times 10^7/\text{ml}$

Calculations are made as follows

Population of parent culture—	$\frac{6.0 \times 10^7}{20}$	$= 3.0 \times 10^6$ bacteria/ml
—O per cell hour—	$\frac{58.2}{6 \times 10^7}$	$= 9.7 \times 10^{-7}$ mm <sup>3</sup>
—O <sub>2</sub> per gm N per hr—	$\frac{58.2}{1.24 \times 10^{-5}}$	$= 4.7 \times 10^6$ mm <sup>3</sup>
Nephelometric index of size—	$\frac{3.7 \times 10^7}{6/8.6 \times 10^7}$	$= 5.3$
—O <sub>2</sub> per nephelometric cell per hour—	$\frac{9.7 \times 10^{-7}}{5.3}$	$= 1.8 \times 10^{-7}$ mm <sup>3</sup>
Nitrogen per cell—	$\frac{1.24 \times 10^{-2}}{6.0 \times 10^7}$	$= 2.1 \times 10^{-10}$ mg

The results of the individual measurements are shown in the figure where they are plotted against the age of the parent cultures. Averages of these values for the several time intervals are given in the table. The values indicated on the graph for cultures at zero time represent the mean of the corresponding values for 18 to 24 hour cultures (*i.e.*, of the cells used for seeding), which accordingly appear twice in the graph. No measurements could be made until populations approaching  $10^7$  cells per milliliter were reached, which occurred near the end of the phase of lag. This probably corresponds with the point of maximum size and activity per cell, as observed by Martin and others (4-6) in proliferating cultures. In several trials with cultures seeded with larger numbers, and examined at 1½ to 3 hours, the activity per viable cell was always much less than the highest values recorded in the table. This finding is in conformity with the observation of Henri (3), that the maximum size attained decreases as the size of seeding is increased.

The curve correlating oxygen uptake with bacterial numbers shows clearly that cells taken from cultures just entering the phase of logarithmic multiplication consume oxygen at six times the minimum rate prevailing near the end of this period. There is no further change as the culture ages. Thus our observations with cell suspensions confirm the findings of previous investigators (4-6), who have used growing cultures, only with respect to the early phases of growth.

*The Average Size of Cells and Rate of Oxygen Uptake in Relation to Age of Cultures of Bacterium coli*

1	2	3	4	5	6	7	8	9	10	11
No of observations	Age of parent culture	Population of parent culture $\times 10^6/\text{ml}$	Gen *	GO <sub>2</sub> †	-O <sub>2</sub> per cell hr $\times 10^{-7} \text{ mm}^3$	-O <sub>2</sub> per gm N per hr $\times 10^6 \text{ mm}^3$	-O <sub>2</sub> per nephelometric cell per hr $\times 10^{-7} \text{ mm}^3$	N per cell $\times 10^{-10} \text{ mg}$	Nos † giving equal turbidity $\times 10^7/\text{ml}$	Nephelometric index of size
	hrs		per hr	min						
3	3	2.8	3.0	35	11.1	5.0	2.1	2.2	0.7	5.3
5	4	36	3.5	35	7.0	4.9	2.0	1.5	1.0	3.7
6	5	220	2.2	35	3.8	4.9	2.0	0.80	2.1	1.9
3	6	450	1.0	35	1.7	4.5	1.7	0.38	3.7	1.0
3	8	830	0.2	35	2.0	4.6	2.0	0.43	3.7	1.0
3	12	1100	<0.1	35	2.1	4.8	1.8	0.42	3.2	1.3
4	18	1300	<0.1	35	2.1	5.1	2.3	0.41	4.1	0.9
4	24	1400	<0.1	35	1.8	5.1	1.6	0.35	3.4	1.1

\* Rate of multiplication in the parent culture by graphic interpolation from values given by the formula  $n/t = \frac{\log b - \log B}{t \log 2}$  where  $B$  = initial, and  $b$  = final numbers, for the observed interval  $t$

† Time required for twofold increase in rate of oxygen uptake in sub-culture

‡ Expressed as numbers per milliliter of saline matching 0.5 per cent CuSO<sub>4</sub>

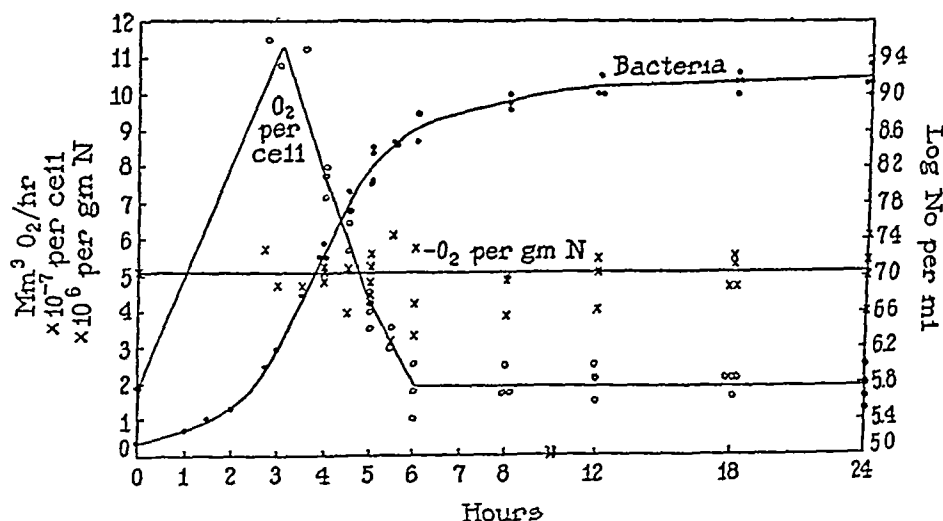


FIG 1 Rates of oxygen uptake per viable cell, and per gram of bacterial nitrogen, in relation to phase of growth of *Bacterium coli* cultures

The continuous decrease in metabolic rate per cell observed by them during the later periods does not appear in our data. We believe that in these earlier experiments the fall in the metabolic rate below the initial value must be attributed to changes in the medium during growth, and to competition within the growing population, especially for oxygen (11-12), and in no way reflects changes in the cells themselves. In our measurements, which were carried out with similar populations in mediums of uniform composition, secondary effects due to changes in environmental conditions have been excluded.

Very different are the results obtained when rates of oxygen consumption are computed in terms of the unit of bacterial nitrogen. On this basis, values for oxygen uptake are constant throughout the period of observation. This suggests that the significant variable in cultures of different age is the average size of cells. Column 9 of the table shows the magnitude of these changes as expressed by the "nitrogen per cell" quotient. This quantitative agreement between changes in oxygen consumption per cell, and the average cell size, may be taken as sufficient evidence that the one is wholly referable to the other. The size indices (column 11) obtained from the nephelometric data are in good agreement with the nitrogen per cell values and lead to the same conclusion.

It is interesting that the time required for a twofold increase in rate of total oxygen uptake of the culture (corresponding to a functional "generation time"), is the same for cells of all ages, as can be seen in the table. This observation suggested another type of experiment (14) in which it was found that multiplication rates of *Bacterium coli* during the early hours of growth are in a large part the expression of changes taking place in the average size of cells. In particular, the phenomenon of lag seemed to be wholly referable to these changes. The manometric findings suggest further that the absolute growth rate is the same for both young and old cells. This conclusion must be made with caution, however, since evidence has been obtained that deprivation of carbon dioxide in the respirometer cultures imposes a new limiting factor on the rate of growth, at least when small inocula are used.

These experiments have not revealed any specific differences between the metabolism of young and old cells which might be described as physiologic youth and maturity. They do not, of course, explain

other differences, such as cataphoretic mobility (8), and susceptibility to unfavorable environment (1), neither of which, however, in our opinion provides adequate basis for the hypothesis of cellular differentiation

Subsequent experiments will be directed toward elucidation of the environmental factors influencing the size of cells, and further correlation between the age of the parent culture and the absolute rate of growth of transplants

#### SUMMARY

1 Measurements of the rate of oxygen uptake per cell in transplants of *Bacterium coli* from cultures of this organism in different phases of growth have given results in essential agreement with the observations of others

2 Correlations of viable count, centrifugable nitrogen, and turbidity, with oxygen consumption, indicate that the increased metabolism during the early portion of the growth period is quantitatively referable to increased average size of cells

3 Indirect evidence has suggested that the initial rate of growth of transplants is not related to the phase of growth of the parent culture

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## ELECTROKINETIC PHENOMENA

### XIII A COMPARISON OF THE ISOELECTRIC POINTS OF DISSOLVED AND CRYSTALLINE AMINO ACIDS\*

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#### INTRODUCTION

The electrokinetic properties of particles in a given medium are due entirely to a surface film which may or may not be chemically similar to the bulk of the particle. Many of the quantitative experiments on electrophoresis have dealt with systems designed to study the influence of the surface film on the bulk of the particle. In these systems the surface films have frequently been proteins and the bulks of the particles have been such diverse substances as quartz, air, and paraffin oil. With few exceptions a film of a given protein adsorbed on inert surfaces has electrokinetic properties quite similar to the electrokinetic properties of the protein when dissolved, or suspended as an amorphous, relatively insoluble particle. Inasmuch as protein films and protein particles have been extensively investigated, the electrokinetic properties of simpler ampholytes like amino acids merit analysis by the same methods used to investigate the electric mobilities and the isoelectric points.

The concept of the isoelectric point was first used to indicate a reference concentration at which the electric mobility of a particle of any sort is equal to zero. Although it was first employed by Hardy (1) to designate the point of reversal of sign of charge of particles of heat denatured albumin, hydrogen ions

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\* These results were presented, in part, at a meeting of the American Physical Society, 1931.

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were not the first used to cause the reversal of the sign of surface charge by electrolytes. In 1880, about twenty years before the experiments of Hardy, Gore (2) investigated electroosmosis through clay diaphragms. He noticed that an alcoholic solution of barium bromide reversed the sign of surface charge. The term isoelectric point later became of importance in connection with dissolved ampholytes. Pauli in 1906 (3) investigated solutions of proteins, and since that time, the isoelectric point has been of importance not only in connection with the characterization of the electrical properties of *surfaces* but also with the conditions under which the net charge of dissolved ampholytes sums up to zero over a time average. Both on the basis of usage and experiment, therefore, a crystal of an amino acid suspended in a liquid may have an isoelectric point as well as the dissolved amino acid itself. Recent discussions frequently consider only the thermodynamic aspects of the isoelectric point of dissolved ampholytes. However, it does not seem, as some of these recent discussions imply, that the term should be restricted to dissolved substances or thermodynamic considerations alone, especially when it is considered that the isoelectric point of particles or of surfaces is usually obtained by a conductance method.

It seems desirable, therefore, to define the isoelectric point as suggested by one of the writers (4) so that the definition includes both dissolved and suspended materials. According to this point of view, the isoelectric point of an amino acid, whether dissolved or crystalline, may be defined as a reference concentration ( $[H^+]$  or  $[Th^{++++}]$ , for example) at which

$$\frac{1}{T} \int_0^T dt \left\{ \sum_1^s n_{i,z_1}(e) + \sum_1^s n_{i,z_1}(-e) \right\} = 0,$$

$$(T \gg \tau)$$

Here  $e$  is the electronic charge and  $n_i$  is the number of ions of the  $i$ -th type, of valence  $z_i$ , at the surface during the time  $dt$ .  $T$  represents the time of observation and  $\tau$  the life period of an ion at the surface. That is, the time average of the total sum of all of the positive and negative ions of the ampholyte is zero in the case of the dissolved ampholyte. Similarly, in the case of any surface or of a large ion, the isoelectric state is that in which the sum of all of the positive and negative charges on the surface over a time average is equal to zero. The conditions relating to the life period of the ion  $\tau$  may be fulfilled by observing a large number of fluctuations during the period of a measurement,  $ze$ , a statistical sample.



A case in point is the cholesterol surface. It has been observed recently by Moyer (5) that the point of reversal of the sign of charge of crystals of cholesterol and ergosterol occurs near pH 3.2. From the point of view of the organic chemist, these sterols can hardly be considered ampholytes. Yet these surfaces in contact with an aqueous medium show ampholytic properties similar to those of adsorbed protein films. They have, therefore, an isoelectric point and can be considered to have amphoteric surfaces which can hardly be described by thermodynamic considerations on the basis of their formulas.

In this communication, a comparison will be made of the isoelectric points of dissolved amino acids obtained by the conventional thermodynamic methods and the isoelectric points of amino acid crystals determined by measuring the electric mobilities of these crystals themselves. The significance of these measurements will be discussed in connection with the constitution of the limits of crystal lattices of amino acid surfaces in their own saturated solutions.

#### HISTORICAL

There is a surprisingly large number of substances which are usually negatively charged when suspended in their own saturated solutions and which show reversal of sign of charge when acids or polyvalent ions are added. Thus it was shown by Perrin (6) for aqueous media, that crystals of phenyl salicylate, naphthalene, sulfur, and metallic oxides may be reversed in sign of charge by hydrogen ions. In addition, zinc sulfate, barium sulfate, chromic chloride as well as silicon carbide, showed similar properties. Since that time, a veritable host of surfaces, especially those composed of silver halides, have been investigated in connection with the effect of isomorphous and homeomorphous ions on the position of the isoelectric point. These crystals have had the added advantage of being studied by both thermodynamic and electrokinetic investigations. In connection with the present report may be mentioned the work of Mukherjee and Iyer (7) who studied certain organic acids and amino acid crystals in their own saturated solutions. These investigators found that phenyl glycine-*o*-carboxylic acid crystals reversed their sign of charge in dilute aqueous hydrochloric acid, whereas crystals of *m*-amino benzoic acid were positive in their own saturated solutions and were reversed by alkali.

The work of Wintersteiner and Abramson (8) showed that surfaces of insulin crystals have a different isoelectric point than adsorbed films of insulin on quartz. By having a sufficient amount of insulin dissolved in the solutions, the crystals apparently adsorb the dissolved protein more rapidly than larger crystals can be built and under these conditions, the crystal surface behaves like the surface of adsorbed insulin.

### Methods

Two horizontal, flat electrophoresis cells, whose dimensions have been previously described (9), were used in the measurements of electric mobility. A detailed description of the electrophoretic methods has been given elsewhere (10-12). All measurements were performed at room temperature (23-27°C). In difficult determinations, measurements were made at successive levels throughout the cell and the resulting parabola integrated (13), but in most cases data were obtained at the stationary levels.

The cystine preparations were obtained from three different laboratories. Tyrosine and aspartic acid were from Eastman Kodak Company and were studied in the form as purchased and after subsequent recrystallization. The melting points of these preparations were all high. Usually the amino acid crystals were finely ground and suspended in the acid or buffer solutions for 24 hours before measurements of pH and electric mobility were carried out. Many experiments were performed after  $\frac{1}{2}$  hour with no observable differences.

The rapid settling of the larger particles to the floor of the electrophoresis cell and the consequent alterations of the surface properties of the glass floor were liable to produce asymmetry of the mobility-depth parabola. In addition, the high conductance of solutions below pH 3 required the use of currents which in some cases appeared to produce turbulence. When such effects were noted, the cells were always cleaned and the measurements repeated. Alternate use of two cells of different dimensions provided a check on the reliability of the data. Many of these difficulties might have been eliminated by the use of the vertical cell described by Abramson, Moyer, and Voet (14), had it been available at the time when these measurements were made.

### EXPERIMENTAL

*Analysis of the  $v$ -pH Curves*—Fig. 1 shows the electrophoretic mobility-pH curve of aspartic acid in solutions of hydrochloric acid. It will be noticed that the isoelectric point of the suspended crystals lies near pH 2.3. The arrow and the cusp in the titration curve indicate the position of the isoelectric point of dissolved aspartic acid at pH 2.9 as calculated from the dissociation constants (15).

Cystine crystals in hydrochloric acid solutions likewise are isoelectric near pH 2.4 (Fig. 2). The three sets of circles are data for the three different preparations. It was desirable to confirm the fact that the isoelectric point of cystine crystals differs in position from the isoelectric point calculated from dissociation constants. Data were therefore obtained in  $M/200$  acetate buffers of constant ionic strength. A few points were also obtained in the same buffer to which

$\mu/5$  NaCl had also been added. Note in Fig 2 that at the isoelectric point of dissolved cystine, as calculated by thermodynamic methods (15), the electric mobilities of the crystals of cystine are rather high and easily measured. The addition of salt to the solution lowers the electric mobility in a way similar to its effect on the mobilities of inert surfaces or of ionogenic surfaces. Although the data obtained in sodium acetate buffers are not sufficiently accurate or numerous for extrapolation to an isoelectric point, the general direction of the smooth curve drawn through the points in Fig 2 is toward

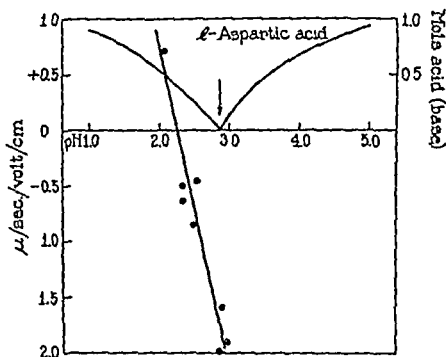


FIG 1 A comparison of the electric mobilities of aspartic acid crystals in HCl solutions and the titration curve of dissolved aspartic acid. The arrow indicates the position of the isoelectric point of dissolved aspartic acid. • = HCl. Smooth curve—titration.

the region designated as the isoelectric point in solutions of hydrochloric acid at pH 2.3. The smooth curve above the abscissa is drawn from the data given by Sano (16) for the solubility of cystine.

In Fig 3 are presented results of measurements of the electrical mobilities of tyrosine. Here a similar comparison is made with the solubility of tyrosine (17) and with the isoelectric points determined for the dissolved tyrosine (15) and the crystals themselves. It is somewhat surprising to note that although the isoelectric point of dissolved tyrosine calculated in the usual way lies near pH 5.7, the

isoelectric point of the crystals falls very close to the isoelectric point noted in the foregoing for cystine and for aspartic acid. As mentioned previously for cystine, tyrosine crystals suspended in  $M/200$  acetate buffers possess a negative charge, and the data indicate that an extrapolation of the smooth curve will pass near pH 2.4. As in the case of cystine, addition of salt, here  $M/8$  NaCl, lowers the electrical mobilities. It is also of interest that in Figs 1-3, there is no point of

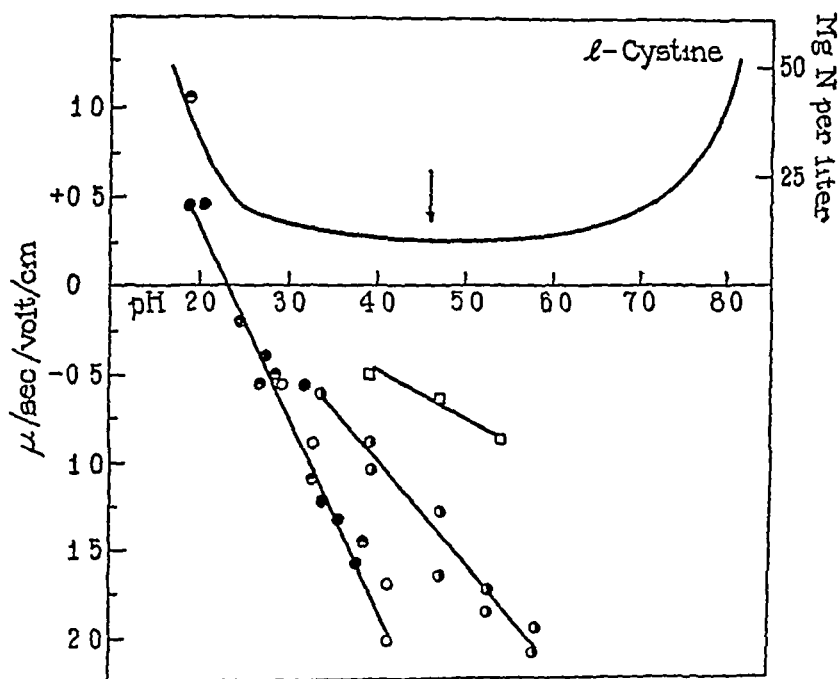


FIG. 2 The electric mobilities of cystine crystals suspended in hydrochloric acid and in sodium acetate buffers. The arrow points to the isoelectric point of dissolved cystine. ●●○ = HCl, ○ =  $N/200$  NaAc, □ =  $N/200$  NaAc +  $N/5$  NaCl. Smooth curve—solubility (Sano).

inflection on the mobility-pH curves of the suspended crystals near the values of pH given for the isoelectric point of the dissolved amino acids.

In a few experiments with cystine the surface properties seem to be reversible, for dilution of the medium initially at a pH below the isoelectric point of the crystals to a value above their isoelectric point

resulted in reversal of sign of charge<sup>1</sup> Data for the construction of smooth electric mobility pH curves for these crystals in HCl solutions are presented in Table I

*Effects of Salts*—As mentioned previously, univalent salts depress the electric mobility with increase of the ionic strength analogous to that observed with other surfaces (18) An increase in the ionic strength of the medium has little if any effect on the position of the isoelectric point of the crystals studied (Figs 2 and 3) It was of

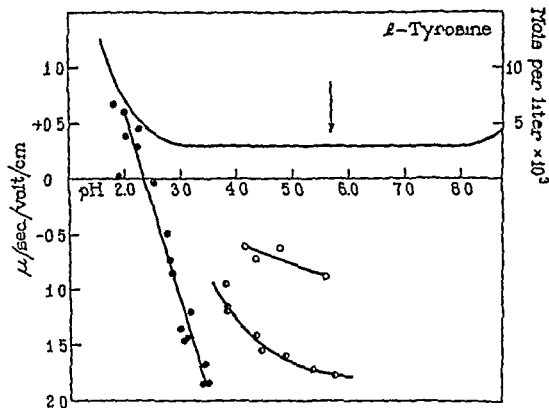


FIG 3 The electric mobilities of tyrosine crystals suspended in hydrochloric acid and in sodium acetate buffers The arrow points to the isoelectric point of dissolved tyrosine ● = HCl ◐ = N/200 NaAc, ○ = N/200 NaAc + N/8 NaCl Smooth curve—solubility (Hitchcock)

<sup>1</sup> Some interesting but rather difficult experiments were performed with amino acid crystals while they were actively dissolving A quantity of finely ground tyrosine crystals was added to sodium acetate buffer and electrophoresis measurements were performed immediately Although sufficient quantitative measurements were not feasible at that time the data obtained indicated that the electric mobilities of dissolving crystals were close to those of the crystals in their own saturated solutions and did not markedly differ from those in Fig 3

interest to study the effect of a polyvalent ion Fig 4 shows the effect of adding low concentrations of  $\text{AlCl}_3$  to a suspension of tyrosine crystals in hydrochloric acid The concentration of aluminum ion is expressed as  $\text{pAl}$  to show that an isoelectric point may be reached

TABLE I

*Data for the Construction of Smooth Electric Mobility-pH Curves for Amino Acid Crystals Suspended in Hydrochloric Acid Solutions*

Aspartic acid		Tyrosine		Cystine	
pH	$\eta$	pH	$\eta$	pH	$\eta$
2.30	0	2.33	0	2.37	0
2.75	-1.50	3.24	-1.50	3.75	-1.50

\*  $\mu/\text{sec}/\text{volt}/\text{cm}$

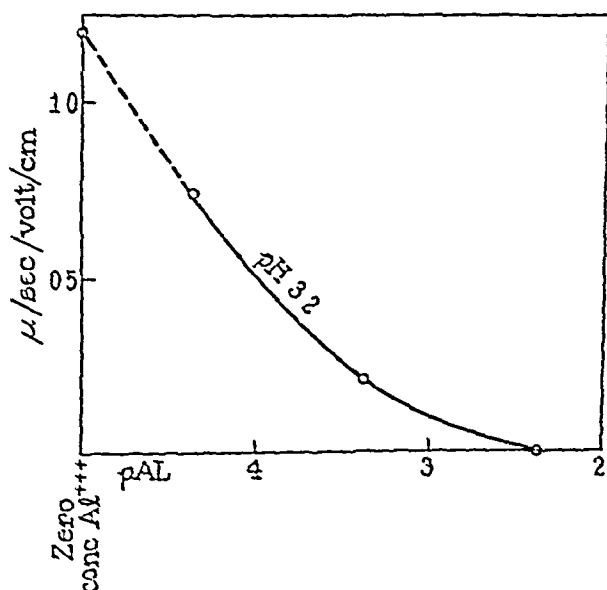


FIG 4 The electric mobility of tyrosine crystals in dilute solutions of  $\text{AlCl}_3$  at approximately constant pH This curve demonstrates that the surfaces of tyrosine are affected by  $\text{Al}^{+++}$  ions in the same way as other surfaces

by addition of  $\text{Al}^{+++}$  ions to a certain activity of aluminum ion just as by adjustment of the solution to a certain pH value an isoelectric point in terms of pH may be attained It may be observed that aluminum ion acts in a conventional way by lowering the electrical

mobilities of the negatively charged crystal surfaces even in very high dilution pH changes due to the addition of the  $\text{AlCl}_3$  were of no importance for the pH rose as the aluminum concentration was increased This would presumably have tended to increase mobilities rather than decrease them Other surfaces, not usually considered amphoteric, such as cellulose (19), may be reversed by polyvalent ions although not by hydrogen ions

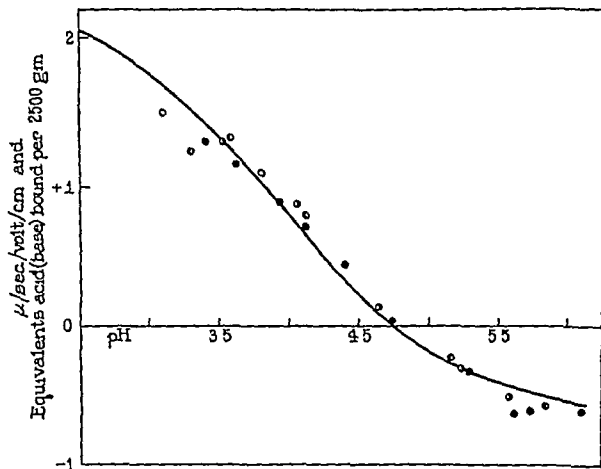


FIG 5 These data demonstrate that amino acid crystals behave very much like inert surfaces in their ability to adsorb gelatin. Evidently the amphoteric properties of the surfaces of the amino acid crystals do not interfere with the adsorption of another ampholyte ● = gelatin adsorbed by quartz, ○ = collodion (Loeb) ◐ = cystine ○ = tyrosine Smooth curve—titration

*Effect of Added Ampholytes*—When crystals of tyrosine or cystine were suspended in solutions of Cooper's gelatin in  $M/150$  acetate buffers, they yielded the same  $\nu$ -pH curve as particles of collodion or quartz when these too were suspended in gelatin (Fig 5) In other words, the gelatin coats the surface of these amino acid crystals just

as it does an inert surface. This lack of specificity of the underlying surface has been observed with most, but not all, adsorbed proteins (20, 21). The smooth curve is the titration curve fitted to the same scale as the mobility data. Similar correspondence between mobility and titration curves has been noted and discussed previously (20, 22).

A series of qualitative experiments on the effect of a simple dissolved ampholyte, glycine, on the isoelectric point of cystine crystals showed no marked influence.

*Comparison with Simple Alkyl Benzenes*—In Fig. 6 are the mobility-pH curves of droplets of *n*-propyl benzene and ethyl benzene in HCl solutions. Within the limits of error the data were the same for two substances. No reversal of sign of charge of the alkyl benzene droplets was noted. At the lowest pH values investigated, their mobility became zero within the limits of error. The surfaces of the amino acid crystals as determined by the slopes of their mobility-pH curves are not markedly different from those of the alkyl benzenes. However, they have one property which markedly distinguishes them from the alkyl benzenes. It is the property which results in reversal of sign of charge in their own saturated solutions at sufficiently low values of pH.

*Effect of Size and Shape on Electric Mobility*—The long needle-like structure of the tyrosine crystals offered an excellent opportunity for the investigation of the effect of needle length and orientation on the electric mobility. Using Eastman Kodak Company tyrosine, it was found that (considering the difficulty of making measurements on particles of this type) crystals and fragments of crystals between 2–30 $\mu$  migrated independent of their length in the electric field. Particles less than 2 $\mu$  long gave a variable mobility, moving more slowly than the others.

Carefully recrystallized tyrosine was powdered and suspended in sugar solution to make a more careful analysis of the effect of particle length on electric mobility. Previous microscopic examination of the unpulverized material in the same medium had shown a uniform suspension of long needles with no very small particles. Examination of the electric mobility revealed that tyrosine needles or fragments of tyrosine crystals from 2–100 $\mu$  migrate independent of their length and



orientation Particles of the order of  $2\mu$  or less show slightly decreased electric mobilities

Two explanations might be offered for the fact that tyrosine particles  $2\mu$  in length show decreased mobilities One of these is the diminution of mobility caused by the size of the particle approaching the thickness of the double layer, that is, a decrease in  $\kappa r$  below a critical value

An alternate explanation, which appears more plausible, is that which has to do with the nature of the crystal itself In order to obtain small fragments of tyrosine needles, the crystals have to be finely ground This results in a change from the natural surface of the crystal formed during crystallization the area of the fractured surface approaches the area of the natural crystalline surface and therefore the electric mobility is changed

#### DISCUSSION

Since x ray diffraction data do not give very exact information in regard to the constitution of the limits of the crystal lattices of the substances investigated when suspended in aqueous media, it is difficult to offer precise mechanisms explaining the behavior of the surfaces of the amino acids under discussion A point of view which is derived from the analysis of the crystal structure and the electrophoretic behavior of the silver halides may be of service in this connection (reviewed by Abramson (23) and Verwey (24)) The reversal of the sign of charge of silver chloride crystals may be visualized by considering that at the limits of the crystal lattice one of each of the six ions of the lattice is lacking for each ion of  $\text{Ag}^+$  and  $\text{Cl}^-$  This results in unsaturated valence forces being made available for reactions with ions opposite in sign of charge Thus, an excess of silver ion reverses the negative sign of charge of silver chloride or silver iodide crystals In a similar fashion, negatively charged barium sulfate crystalline surfaces may be reversed in sign of charge We have seen that hydrogen ions alone do not reverse the sign of charge of simple alkyl benzenes We must invoke, therefore, some mechanism essentially connected with the constitution of the crystal lattice of the amino acid crystal to explain the apparently simple reversal of the sign of surface charge The only mechanism which has occurred to

us is one which would assume that reversal of the sign of charge is, in part, brought about by the deposition in the lattice of the isomorphous ions of the positively charged dissolved amino acids themselves. In this way, in the case of tyrosine, for example, if the pH is decreased and more tyrosine goes into solution, not only will there be an increasing number of ions available for adsorption, but also a markedly increased number of positive tyrosine ions. It is possible that these tyrosine ions may fit into the lattice and produce a reversal of sign of charge in the same way that silver ions may produce reversal of sign of surface charge of a crystal of silver chloride. In this connection, observe the slopes of the mobility-pH curves of the crystals of amino acids in Fig 6 where all three are given for comparison. At first glance, it would seem that reversal of sign of surface charge occurs more rapidly in those instances where the increased solubility occurs most rapidly, that is, the slope of the tyrosine curve is greater than the slope of the cystine curve, and is less than that of the aspartic acid. However, the rapid change in the mobility of the alkyl benzenes with pH does not support this point of view. Not all crystalline organic surfaces, however, would fall into the category established for the silver halides or for the point of view given here for the amino acid crystals. Thus Moyer (5) has shown that the crystal surfaces of cholesterol appear to be simply reversed in sign of charge by hydrogen ions. From the point of view of structural chemistry, there are no groups which react with hydrogen ions to produce reversal of sign of charge, nor is cholesterol sufficiently acid in character to account for its isoelectric point at pH 3.2, the point given by Moyer for his purest preparations. One might suppose that the order of increasing slopes of the mobility-pH curves (Fig 5) would follow the order of pH values of the corresponding isoelectric points for dissolved amino acid molecules. Thus an amino acid whose isoelectric point (in the dissolved state) was high on the pH scale would begin to have its mobilities reduced by its adsorbing positively charged ions at a pH value where crystals of another amino acid, with dissolved molecules isoelectric at a lower pH, would be scarcely affected. As seen from Fig 6, however, the results do not bear out this simple picture for the order of the slopes is not the same as the order of the corresponding isoelectric points. It will be necessary to include in the development of

a complete theory of the effect of pH on the electric mobilities of crystalline amino acids, the effect of adsorbed hydrogen ions in addition to the entrance of dissolved amino acid molecules into the surface, even though the effects of hydrogen ions may be less important than isomorphous ions

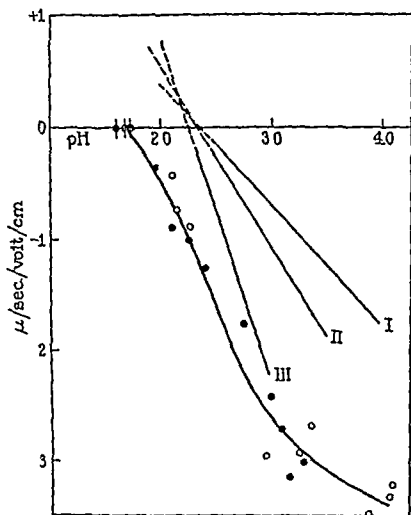


FIG 6 A comparison of the electric mobilities of emulsion droplets of simple alkyl benzenes with those of the surfaces of crystalline amino acids  $\circ$  = *n* propyl benzene,  $\bullet$  = ethyl benzene, I = cystine (crystal) II = tyrosine (crystal), III = aspartic acid (crystal)

In the case of the surfaces of the keratins, such as wool (25), hair, skin, and finger nails (26), the electrokinetic properties are probably influenced more than adsorbed proteins by the constraints occurring at the surface of the crystal lattice. Since in the case of the keratins the elongated protein molecules are arranged side by side and held together by S—S linkages for the most part (27, 28), it seems likely that the constraints imposed on the reacting groups of the sur-

face are not so important as they would be in determining the electrokinetic properties of a truly crystalline amino acid and that the isoelectric point found by Harris represents the ionization of the protein molecules fairly closely. Goddard and Michaelis (28) have presented values for the flocculation maximum of wool brought into a dissolved state by thioglycolic acid and other reducing agents. Data on the electrokinetic properties of these dissolved keratins would be of interest. A simpler case is afforded by the experiments of Harris (29) on silk fibroin. In this protein the elongated molecules in the crystallite are not held together so tightly as in keratin since peptization may be readily produced. S—S linkages are also lacking. Harris has found that the electric mobilities of particles of ground-up silk fibroin are identical with the mobilities of silk fibroin adsorbed on quartz particles. The isoelectric point was the same for both crystallite surface and adsorbed protein surface, lying near pH 2.5.

Comparison should be made between these results and those of Wintersteiner and Abramson (8), discussed in the foregoing, for crystalline and amorphous insulin surfaces, an example of a more truly crystalline protein. It is probable that the rearrangement occasioned by the transformation of a spherical, dissolved protein such as egg albumin or insulin into the crystalline state would produce effects which serve to distinguish surfaces of this type from the keratin surface.

A better understanding of the nature of the surfaces of amino acid crystals and the surfaces of crystallites like keratin must await the investigation of the surfaces of long-chain amino acids and polypeptides.

#### SUMMARY

1 Although the isoelectric points of dissolved cystine, tyrosine, and aspartic acid molecules lie at widely differing pH values, the isoelectric points of the surfaces of these substances in the crystalline state are all near pH 2.3. This was found to be true in solutions of hydrochloric acid and in acetate buffers of approximately constant ionic strength.

2 When suspended in gelatin, tyrosine and cystine crystals adsorb

the protein and attain a surface identical in behavior with gelatin-coated quartz or collodion particles

3 Aluminum ions at low concentrations reduce the electric mobilities of tyrosine crystals to zero in a manner analogous to their effect on other surfaces

4 Alkyl benzene droplets also have their electric mobility reduced to zero at low pH values but, unlike the amino acids, a change in sign was never noticed

5 The mobility of tyrosine crystals is independent of crystal length between 2 100 $\mu$  Below this size the mobilities are decreased

6 These results are discussed in connection with the concept of the general definition of the isoelectric point and the behavior of certain insoluble proteins such as wool and silk fibroin

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# THE ADSORPTION OF PROTEINS AT OIL-WATER INTERFACES AND ARTIFICIAL PROTEIN-LIPOID MEMBRANES

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Emulsions of oil in water can theoretically be thermodynamically stable only if the interfacial surface tension  $\gamma$  between the two phases is zero. This follows from the fact that the pressure in a droplet of radius  $r$  exceeds that in the surrounding liquid by an amount

$$\Delta p = 2\gamma/r \quad (1)$$

When two drops coalesce the pressure within the drops decreases and there is a decrease in free energy.

If a liquid is heated to its critical temperature  $\gamma$  vanishes when its separate phases disappear. Similarly if phenol and water are mixed and heated to the critical solution temperature the two liquids become miscible when  $\gamma = 0$ . These considerations would seem to indicate that all emulsions must be inherently unstable or metastable since on the one hand stability requires  $\gamma = 0$  and on the other hand  $\gamma = 0$  requires that there shall be only one phase.

Closer examination, however, shows that even when  $\gamma = 0$ , drop leters may be stable and there may be no tendency for the separate phases to disappear.

If an insoluble non volatile substance is spread as a monolayer at an air water interface of surface area  $S$ , the lowering of surface tension may be looked upon as being due to a spreading force  $F$  exerted by the adsorbed molecules as the two dimensional analog of a pressure. Here  $F$  is defined by

$$F = \gamma_0 - \gamma \quad (2)$$

where  $\gamma_0$  is the surface tension of the pure solvent and  $\gamma$  is that of the surface covered by the monolayer.

In general  $F$  depends upon  $\sigma$ , the number of molecules/cm<sup>2</sup>, in the monolayer and upon the temperature. The relation connecting these three quantities may be called an equation of state, and is the two dimensional analog to the equation which relates the pressure with the density and temperature of a gas.

If the free surface  $S$  is altered, as by moving a barrier, the total number of adsorbed molecules  $S\sigma$  remains constant. Thus for insoluble adsorbed substances  $F$  depends on  $S$  in a manner that can be calculated from the equation of state. In general  $F$  increases rapidly as  $S$  decreases.

A substance which lowers the surface tension of water but is appreciably soluble in water also forms an adsorbed film on the surface, and  $F$  is still related to  $\sigma$  and  $T$  by an equation of state. But now if the free area  $S$  is decreased  $S\sigma$  does not remain constant, for some of the adsorbed molecules can go into solution. The relation between the properties of the adsorbed film and the concentration  $c$  of the dissolved substance in the solution is given by Gibbs' equation

$$dF/d \ln c = \sigma kT \quad (3)$$

By combining this with the equation of state so as to eliminate  $F$  it is possible by integration to obtain an "adsorption isotherm" which gives  $\sigma$  in terms of  $c$  and  $T$ . On the other hand, if we eliminate  $\sigma$  we obtain an equation which we may call the "force-isotherm" that expresses  $F$  as a function of  $c$  and  $T$ .

With a solubility as great as one part per million, the amount of solute in the water phase is usually far greater than the amount adsorbed and therefore if  $S$  is changed  $c$  does not change appreciably and  $c$  remains constant. Thus even a slight solubility may have a profound effect on the relation between  $F$  and  $S$ .



Let us now take the case in which the substance adsorbed at the surface of the oil drops is insoluble in both the water and the oil. Consider a single droplet that has  $N$  molecules adsorbed on its surface  $S$ , so that

$$\sigma = N/S \quad (4)$$

With a sufficiently large value of  $N$  it is possible that  $F$  may be so great as to make  $\gamma = 0$ , which will occur if  $F = \gamma_0$ .

It is now no longer necessary that the two phases should disappear. Consider, for example, a droplet of spherical form for which  $\gamma = 0$ . If the drop becomes distorted its area increases, and therefore  $\sigma$  and  $F$  decrease and  $\gamma$  becomes positive. Any distortion of the drop from the spherical form brings into existence a force which causes the drop to return to spherical form.

If the amount of adsorbed substance is increased beyond that needed to make  $F = \gamma_0$ , or  $\gamma = 0$ , for the spherical drop, the drop will automatically increase its surface by assuming some non spherical form, but  $\gamma$  remains zero.

According to this theory it seems possible to explain the existence of stable oil water emulsions having an insoluble emulsifying agent adsorbed on the surfaces of the drops which have zero surface tension.

### *Protein Monolayers on Water*

Henri Devaux in 1903 found that egg albumin forms insoluble monolayers at an air water interface. In a recent paper<sup>1</sup> he described experiments to measure the degree of insolubility. Egg albumin monolayers formed either by spreading from a solid fragment of the substance or from dilute solutions can be reversibly compressed to one half area.

Since this protein is normally soluble in water, one would expect from equation (3) that the solubility would be enormously increased by compressing the film. For example, let us consider a protein monolayer at  $F = 0$  having an area of 10 sq. meter per mg. and assume a molecular weight of 35,000. This gives  $\sigma \approx 1.7 \times 10^{12}$  molecules/cm<sup>2</sup>, and equation (3) at 20°C gives  $d \ln c = 220 dF$ . A compression of the film from  $F = 0$  to  $F = 15$  should thus increase the solubility by a factor of 10<sup>25</sup>.

<sup>1</sup> Devaux, H., *Compt. rend. Acad. sc.*, 1935, 201, 109.

Actually, however, the monolayer at all pressures seems to be wholly insoluble. In fact, the process by which soluble egg albumin spontaneously spreads to form a monolayer on the surface appears to be irreversible. Devaux showed that, after compression of the monolayer to about one-fifth of its area, the area returns nearly to its original value when the force is removed. With a compression to less than one-fifth area, there is a gradual loss of the power to expand completely and one can then observe minute folds in the film which become more pronounced the greater the degree of compression. However, even if the film is compressed to one-twelfth area or less there is no evidence of any solution in the underlying water. By crumpling the film into a very narrow strip it can be lifted off as a thread or fiber by means of a platinum wire. If this is placed, either wet or after drying, upon a fresh surface of water dusted with talc it is seen that there is no tendency whatever for this substance to spread.

Experiments by Mr. Schaefer in this laboratory with egg albumin, pepsin, insulin, and other proteins have confirmed this complete insolubility of protein monolayers.

Devaux,<sup>2</sup> with very dilute aqueous solutions of egg albumin in a tray 2 or 3 mm deep, found that at concentrations greater than  $10^{-7}$  parts by weight a monolayer is gradually formed which is thicker than that which corresponds to  $F = 0$ . The diffusion of the protein to the surface at these low concentrations is very slow, so that in each experiment the solution was allowed to stand for 24 hours before the monolayer was tested. A definite portion of the monolayer between two barriers was then allowed to expand by moving these barriers apart, until the force  $F$  fell to zero as indicated by the action of talc when blown upon lightly. It was found that the higher the concentration of the solution the greater the expansion ratio. Multiplying this ratio by  $11 \text{ \AA}$ , the thickness of the monolayer at  $F = 0$ , Devaux obtained the values given in the second column of Table I. With solutions more concentrated than  $10^{-6}$  it was necessary to replace the protein solution under the film by pure water in order to avoid the diffusion of a new supply of protein to the surface during the expansion of the film.

<sup>2</sup> Devaux, H., *Compt rend Acad sc*, 1935, 200, 1560

If we take Philipp's value of 1.15 sq m/mg for an egg albumin monolayer at  $F = 0$  and use the ratio of expansion as found by Devaux, we obtain the data in the third column.

The data in the last column are the approximate values of  $F$  needed to compress egg albumin monolayers to the various thicknesses given in column 2. These are rough determinations by Mr. Schaefer made with a commercial grade of egg albumin.

It is evident that although the reaction between a protein solution and a monolayer is irreversible the pressure  $F$  builds up in 24 hours to a value that varies with the concentration of the underlying solution. A kind of pseudoequilibrium results.

TABLE I  
*The Formation of Monolayers on Egg Albumin Solutions*

Protein concentration parts by weight	Film thickness $\text{\AA}$	m <sup>2</sup> /mg	$F$
$10^{-2}$	46	0.28	33
$10^{-3}$	30	0.42	29
$10^{-4}$	24	0.53	25
$10^{-5}$	20	0.63	22
$5 \times 10^{-7}$	17	0.75	18
$4 \times 10^{-8}$	13	0.97	4
—	11	1.15	0

The rate of change of  $F$  with  $\log c$ , as given in Table I, corresponds to a value of  $\sigma \approx 4 \times 10^{12}$  molecules/cm<sup>2</sup> according to equation (3), in the range of  $c$  from  $10^{-2}$  to  $10^{-8}$ . Combining this with the data in the third column, we find that the variation of  $F$  is as great as if the molecular weight of the proteins ranged from 2000 to 5000. However, since equation (3) is derived from thermodynamic principles, there is no reason to expect it to be applicable to the irreversible phenomena of Table I, so the foregoing calculation is of doubtful significance.

#### *Protein Monolayers at Oil-Water Interfaces*

The property of proteins of forming insoluble monolayers should make these substances particularly suitable for testing our hypothesis that stable emulsions can be formed containing droplets having zero surface tensions.

A third paper by Devaux<sup>3</sup> describes experiments which have an intimate bearing on this problem. He took a solution of  $10^{-5}$  parts by weight of egg albumin in water in a flask, added some pure benzene, and shook it. With this concentration of protein the droplets of benzene rise, join with one another, and form a separate phase floating on the water. The protein is adsorbed at the interface. If the amount of protein is sufficiently small in relation to the area of the interface, the interfacial surface appears clear and brilliant. By increasing the amount of protein or decreasing the area of the interface, the surface becomes suddenly dull and cloudy, and when agitated the cloudiness varies irregularly over the surface. Devaux determined the amount of protein necessary to produce this peculiar appearance, and concluded that the amount needed is just sufficient to cover the interface with a monolayer of from 9 to 22 Å thickness, depending upon the pH of the solution. The cloudy appearance of the surface when the amount of protein exceeded a definite limit is due to the presence of minute folds of the protein membrane in the interface. The presence of folds at the interface should occur when  $\gamma = 0$  or when  $F = \gamma_0$ .

We have repeated these experiments of Devaux, using a long tube about 25 cm long and 3 cm in diameter. This tube is filled about one-third with protein solution and about one-quarter with benzene. After shaking, the tube is held in a horizontal position until the phases separate. By tilting the tube gradually toward the vertical position, the interfacial area can be decreased progressively until at a critical angle the cloudy appearance described by Devaux appears. We have also tried the Devaux experiment using light mineral oil and water and obtained substantially the same results.

With higher concentrations, such as  $10^{-3}$ , the droplets of benzene do not readily join together. They obviously have a very low surface tension, and a few drops are often seen which are drawn out into sharp points with a cloudy membrane over their surface, just as would be expected if the interfacial tension were zero. However, even with concentrations of protein considerably above the critical value most of the droplets seem to have definitely a positive surface tension.

<sup>3</sup> Devaux, H, *Compt rend Acad sc*, 1936, 202, 1957

Since  $\gamma_0$  for benzene water is about 35 dynes/cm, the adsorbed protein film at the interface must be very highly compressed, and it seemed possible that at such high pressures irreversible changes occur like those observed at air water interfaces causing  $F$  to decrease gradually below  $\gamma_0$ . It would therefore seem better to use a pair of liquids that have a lower interfacial tension than water benzene.

### *Protein Membranes*

For many biological problems it would be desirable to have one or two monolayers of protein forming a membrane between two aqueous phases. Such a membrane may be expected to have some of the properties of a cell surface. It would be interesting to study its electrical properties and its permeability for various substances.

Mr. Schaefer and one of us, some months ago, made preliminary experiments in the effort to obtain such membranes. We found that a platinum wire loop (8 mm diameter) or a plate perforated by small holes, when lifted up through water on which there is a monolayer of egg albumin subjected to a pressure of 30 dynes/cm or more, retained a film like that from a soap solution. These films could frequently be dried in air without rupture. They were very difficult to see, for they reflected practically no light, as their thickness was far less than the wave length of light. Under the microscope with dark field illumination they became visible when punctured, for light was scattered from the edges of the hole in the protein film. Films of this kind in air do not seem to meet the needs of the biologist. We were not able to immerse such films in water without rupturing them.

Attempts to lower a platinum loop or perforated plate into water covered with a protein monolayer seemed in some cases to give membranes, but they were so fragile that the method did not seem promising.

We have recently attempted to build protein membranes by lowering loops or perforated plates through the interface between benzene and water at which there is an adsorbed protein monolayer, at a concentration which gives approximately  $\gamma = 0$ .

A 0.1 per cent solution of egg albumin in water in a beaker was covered with a layer of benzene. After a short time, on slightly agitating the beaker, the characteristic cloudy appearance of the inter-

face was seen. On lowering a wire loop through the interfacial film a membrane was formed which lasted at best about 20 seconds. It seemed that when the protein monolayers on the opposite sides of the loop came into contact by the gradual up-flow in the intervening benzene film, the membrane ruptured. Observation of the meniscus at the benzene-water interface during the lowering of the loop proved, however, that the interfacial surface tension was not zero. If it had been zero, there should have been no meniscus.

During the lowering of the loop through the interface there must be a radial in-flow of the protein monolayer toward the loop. These highly compressed protein films seem to possess a certain degree of rigidity like those on the surface of water, so that the surface tension may not equalize itself over the whole surface.

If the film is stretched by lowering the loop, the taking up of new protein from the underlying solution seems to be a very slow process especially when the film is highly compressed. For example, Devaux found that fragments of solid egg albumin would build up films of a thickness of 65 Å after 24 hours. Some experiments that we have made showed that within 10 minutes the presence of a solid fragment of egg albumin on the water surface raises  $F$  to about 18 dynes, which would correspond to a film of a thickness of only about 17 Å. The rate of arrival of protein molecules into the monolayer from the underlying solution probably slows up very rapidly as the thickness of the monolayer increases.

It seemed to us therefore that it would be desirable to have an interfacial film which had a surplus of folds in it before we attempted to build a membrane. We found that this could be done by lowering a chromium plated slide through the benzene-water interface, holding it under the water for a short time, and lifting it out again. The surface of the plate had been polished and made hydrophobic by covering it with molten ferric stearate and rubbing vigorously with a clean cloth to remove all but a monolayer of stearate.

The effect of lowering the plate through the interface was to carry down a film of benzene and to stretch the interfacial film, thus decreasing its thickness so that new protein could rapidly be taken up by the film. On raising the plate, the protein monolayer which had been carried down by the plate returned to the interface between the bulk

phases of the water and the benzene, producing a circular area in the middle of this interface which was obviously greatly folded and far more cloudy than the surrounding surface. This *prestretching* of the surface gives an interfacial area much larger than the apparent one. After 10 to 20 seconds, however, the folds that were produced in this way seemed gradually to disappear, leaving the interface in its original condition. If the platinum wire loop was lowered through the interface, within a few seconds after prestretching, the membrane formed on the loop persisted for about 1 minute. While the wire frame was being lowered the folds produced by the prestretching were used up during the formation of the first part of the membrane. During this time there was practically no meniscus. The remaining portion of the membrane was formed under slight tension, as indicated by the meniscus, and this was probably responsible for the subsequent rupturing. When the membrane did break it disappeared completely.

In cell surfaces, besides proteins, there are present such substances as sterols and lecithin, which may be responsible for the stability. We tried some experiments therefore in which we added cholesterol to the benzene, but without appreciably improving the results.

The addition of 0.5 mg. of lecithin per cc. of benzene greatly improved the results. A layer of this solution over a 0.1 per cent solution of egg albumin in water gave membranes which lasted several minutes without rupturing and when they finally broke it was seen that there was only a small hole near the center of the film. The hole, however, grew rapidly in size until the Devaux crinkling effect was seen around the edges, indicating that the interfacial surface tension had then become zero. A partly ruptured membrane having a hole covering one fifth of its area remained unchanged for several hours.

The rupturing of these membranes was overcome by prestretching the interfacial film between the dilute lecithin solution and the aqueous protein solution. The hydrophobic chromium plate is lowered into the interfacial film for about one half minute. On withdrawing it there is a large circular area on the interface having a great number of folds which show little tendency to disappear, indicating that the lecithin greatly stabilizes the interfacial film and prevents it from contracting or collapsing. If a wire loop is dipped into the vicinity of these folds, a membrane is formed which, although covering the

total area of the wire loop, shows the Devaux cloudiness. These membranes if undisturbed show no tendency to rupture. We made several of these membranes on separate wire loops suspended in the water phase from the sides of the beaker. By perfusing the vessel with a very slow stream of distilled water, the benzene, the interfacial film, and the protein solution were slowly washed away, leaving the intact membranes in pure water. The benzene which was originally between the two protein monolayers was presumably dissolved by the water, since it is appreciably soluble in water. The membrane probably therefore consists of two layers of protein separated by one or two monolayers of lipid.

The failure to obtain good protein membranes without the lecithin and the disappearance of the folds in the interface produced by pre-stretching are probably caused by a gradual crumpling of the protein monolayer at the interface due to formation of cross linkages between the molecules, resulting in a kind of denaturation of the film. The difficulty thus seems to be due to the fact that  $F = 35$  is too large a spreading force for a pure protein to stand indefinitely. A pair of liquids showing lower interfacial energy might solve the problem. The lecithin, however, seems to enable the film to withstand this pressure.

It seems possible that by the addition of a substance that would lower the interfacial tension a similar result might be obtained. We tried the effect of the addition of ethyl alcohol, but we did not find any improvement in the protein films by its use. This may well be due to a displacement of the adsorbed alcohol by the protein.

5 ml. of saturated cetyl sulfate solution injected into 100 ml. of the protein solution under the benzene made it much easier to get the Devaux effect and it persisted longer. Traces of such substances may facilitate the building of membranes. Using an egg albumin solution without added substances the Devaux effect produced by gently tapping the beaker was very much more pronounced after the protein solution had been allowed to stand for 24 hours in contact with the benzene. This suggests that after long periods of time the value of  $F$  can finally rise to 35 in spite of the initial tendency to collapse.

#### *Stabilization of Oil-Water Emulsions by Proteins*

Observations of the surface tensions of globules of benzene in dilute protein solutions seemed to indicate that in most cases the surface



tension was not quite zero. The difference in density between the two liquids, however, somewhat interfered with the interpretation of these results. We therefore added enough carbon tetrachloride to the benzene to make the density nearly that of water, and this was then shaken in about twice its volume of water. Upon addition of a few drops of 1 per cent egg albumin solution the tube was gently shaken and allowed to stand. The hydrocarbon phase remained suspended almost motionless in the form of large drops throughout the aqueous phase. Some of these drops were not even approximately spherical, but were of very irregular shape, showing that the surface tension was actually zero. In some cases it was found that a large drop of the hydrocarbon phase contained a large drop of water inside it. It would seem that by perfusing such a double drop with water it would be possible to dissolve out the benzene and the carbon tetrachloride, leaving a double shell of protein which would have considerable resemblance to a cell wall.

We believe that the technique we have described for building protein membranes across holes in plates may be useful to the biologist in the study of the properties of these membranes. Such a plate having a hole covered by a membrane could form the partition between two separate aqueous solutions, so that permeabilities and conductivities, etc., could be studied.



# TRANSVERSE ELECTRIC IMPEDANCE OF THE SQUID GIANT AXON\*

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The alternating current impedance of a nerve may be measured in a moist chamber between electrodes placed along its length, as is usually done for excitation and action potential studies. The impedance of several nerves has been measured in this manner (Krüger, 1928, Lullies, 1930, 1934, Cole and Curtis, 1936). Labes and Lullies (1932) have interpreted the data of the latter on the basis of a core-conductor model with a double layer and a diffusion capacity at the axon membrane.

A more satisfactory analysis of this "longitudinal" impedance would involve not only the flow of current into and along a single axon, but also the electrical properties of the other axons in the nerve trunk and the current flow through and around them. Such an analysis would be difficult and probably too cumbersome to be useful, so it is expedient to look for an experimental technique where the results can be analyzed more easily.

A length of nerve may also be laid between two long parallel electrodes so that the direction of the current flow will be primarily perpendicular to the axon axes. For a homogeneous nerve, this "transverse" impedance may be readily derived theoretically and in a simple algebraic form (Bozler and Cole, 1935, Cole and Curtis, 1936). On this assumption of homogeneity, measurements show that the axon membrane has a polarization impedance. Unexplained effects analogous to polarization impedance are found at metal electrolyte interfaces, in imperfect dielectrics, and internal viscosity of solids, but the

\* Aided by a grant from The Rockefeller Foundation

equation which describes them all is purely empirical and the use of the term polarization impedance is an admission of ignorance. For an inhomogeneous nerve, the calculations are more involved (Cole and Curtis, 1936) but a distribution of static membrane capacities among the axons can be assumed which will also explain the data. However, the necessary range of capacities is so great as to seem quite unreasonable since the membrane capacities of a variety of other cells are within a factor of three of each other. Both of these interpretations of the transverse impedance data on whole nerves are rather unsatisfactory since they do not uniquely determine the characteristics of the individual axon.

It is therefore necessary to further simplify matters by making transverse measurements on a single axon, but this was not considered possible until we were introduced to the squid giant axon by Dr. John Z. Young (1936). We are also very much indebted to him for his assistance in preliminary experiments which were made during the summer of 1936 at the Biological Laboratory, Cold Spring Harbor, Long Island.

### Procedure

The Woods Hole squid, *Loligo pealii*, was used and the dissections were made at a temperature of about 10°C. The hindmost stellate nerve was dissected free between the stellate ganglion and the point where it enters the mantle muscle and the small fibers teased away from the giant axon *in situ*. The axon was then tied off at the proper length with fine silk threads, cut free at both ends, and transferred to the conductivity cell. In large animals, these axons can be dissected clean for a length of over 5 cm and they have diameters from 0.5 to 0.6 cm or sometimes larger. With careful dissection and proper treatment they can be kept excitable for several hours after excision. Early measurements were made at about 2°C but it was later possible to work with excitable axons at room temperature.

The conductivity cell constructed for these measurements is similar to one which was used for the transverse impedance measurements of *Nitella* (Curtis and Cole, 1937). Since the sea water in which the axons were measured has a much lower specific resistance than any of the electrolytes used for *Nitella*, larger electrodes were necessary to avoid serious electrode polarization difficulties. This cell, shown in Fig. 1, was built up of glass and de Khotinsky cement with a long rectangular groove in the top for the axon. At the center of each side, the wall of this groove was cut away for a length of 5.6 mm and a depression ground

leading to a platinized platinum electrode 12 mm by 5 mm cemented to the glass. The top surface of the cell was then ground flat.

The entire cell was filled with sea water and covered with a flat glass plate after the axon had been placed on the plateau remaining between the electrodes, and in the grooves at either end of it.

There was then a considerable amount of sea water whose resistance was in series with the impedance of the axon, but the whole cell may be considered as made up of an inactive part whose resistance is unaffected by the axon and an active part which is equivalent to the cell used for *Nitella* measurements. The cell constants of these two parts were determined by equation (1) from measurements of the resistance with a series of small glass rods in place of the axon. As a check, a few axons were measured in a small electrode cell of the type used for *Nitella* measurements. The latter data are not very satisfactory, but to within the limit of error they agree with the measurements made with the large electrode cell.

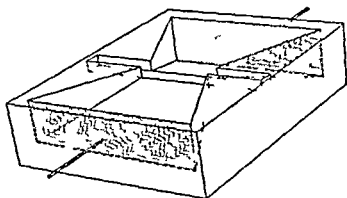


FIG 1 Measuring cell for transverse impedance of a single axon

As soon as the axon was in place the cell was connected to the Wheatstone bridge and the excitability tested as follows. The bridge was balanced with a small voltage, at a low frequency 30 to 200 c (cycles per second), a cathode ray oscillograph being used as null point detector. The amplified bridge output was connected to the vertical deflecting plates. The oscillograph figure was then an ellipse which, at balance, became a horizontal straight line. As the voltage input to the bridge, and consequently the current through the axon, was increased a small hump appeared on the oscillograph pattern. This hump increased with the increasing input until the action potential suddenly appeared in its place.

### Measurements

The general construction and operation of the alternating current Wheatstone bridge used for these measurements has been described in detail (Cole and Curtis, 1937).

The bridge input was reduced to a small fraction of that necessary to stimulate and the impedance measured as the parallel resistance,  $R_p$ , and capacity,  $C_p$ , at eleven frequencies from 1 to 2500 kc (kilocycles per second). The excitability was tested again at the end of the frequency run and until the axon failed to respond. Then another frequency run would be taken. In many cases, the axon was left in place until, after several hours, the low frequency resistance slowly fell to a low value and the parallel capacity disappeared.

The axon was measured and removed from the cell, which was then filled with sea water. Another frequency run was taken which determined the electrode polarization corrections at the lowest frequencies and the capacity of the empty cell at higher frequencies.

The impedances were then computed as series resistance,  $R_s$ , and resistance,  $X_s$ , at the different frequencies and plotted as the impedance locus (Cole, 1928). This locus for a typical axon is shown in Fig. 2.

### *Transverse Impedance Theory*

The equation for the specific impedance,  $z$ , between opposite faces of a nearly square cell with a cylindrical axon at the center, as given by Rayleigh (1892), and used for the *Nitella* measurements, is the same as for a uniform suspension of uniform parallel cylinders

$$\frac{1 - r_1/z}{1 + r_1/z} = \rho \frac{1 - r_1/z_2}{1 + r_1/z_2} \quad (1)$$

where  $r_1$  is the specific resistance of the medium,  $\rho$  is the fractional part of the volume of the cell occupied by the axon, and  $z_2$  is the equivalent specific impedance of the axon. This latter is given by

$$z_2 = r_2 + z_3/a \quad (2)$$

(Cole, 1928), where  $r_2$  is the specific resistance of the axon interior,  $z_3$  the membrane impedance, and  $a$  the axon radius. As has been shown when  $z_3$  is a polarization impedance with a constant phase angle independent of frequency, the impedance locus is a circular arc with its center below the resistance axis (Cole, 1928, 1932). The phase angle,  $\phi$ , of  $z_3$  is then half the angle between the radii to the intercepts of the arc on the resistance axis.

It can be further shown that the membrane capacity per unit area,  $c_M$ , can be computed directly from equations (1) and (2) or more simply by the equation (Cole and Curtis, 1936),

$$c_M = \frac{k}{a\omega r_0} \frac{1 - r_\infty/r_0}{1 - (r_1/r_0)^2} \quad (3)$$

$k$  is the "active" cell constant,  $r_0$  and  $r_\infty$  are the circular arc extrapolated resistances at zero and infinite frequency, and  $\omega$  is  $2\pi$  times the characteristic frequency, for which the series reactance is a maximum. Since the capacity of a polarization impedance varies with frequency and  $c_M$  has the value given by equation (3) at the characteristic frequency, the capacities in all cases have been calculated at 1 kc.

The internal resistance of the axon  $r_2$  may be expressed in terms of the resistance of sea water,  $r_1$ , and  $r_\infty$  from equations (1) and (2), or by

$$r_2 = \frac{r_0 r_\infty - r_1^2}{r_0 - r_\infty} \quad (4)$$

The resistance for a unit area of membrane  $r_3$  is computed (Cole, 1937) from the difference between the actual volume concentration  $\rho_n$  and the volume concentration  $\rho$  which is computed at low frequency by equation (1) considering the rest of  $z_3$  to be infinite

$$r_3/a = r_1 \frac{(2 - \Delta)}{\Delta} - r_2,$$

where

$$\Delta = (\rho - \rho_n)/\rho$$

### *Data and Interpretations*

It is seen from Fig 2 that equation (1) is an adequate representation of the data on this axon over most of the frequency range, when the membrane has a polarization impedance with a phase angle of  $79^\circ$ . The phase angles for other axons varied from  $64^\circ$  to  $85^\circ$  with  $76^\circ$  as an average value. From equation (3), it was found that the membrane capacity at 1 kc for the axon of Fig 2 was  $0.97 \mu\text{f}/\text{cm}^2$ . Values were obtained from 0.66 to  $1.60 \mu\text{f}/\text{cm}^2$  and the average of twenty two experiments was  $1.07 \mu\text{f}/\text{cm}^2$ . No significant change of these quantities with temperature was observed.

In general, the high frequency points fit the circular arc no better than in the data plotted, but from equation (4) and the values of  $r_{\infty}$  extrapolated by the arc, internal resistances are calculated from 1.5 to 6.9 times the resistance of sea water with an average of 4.2. Considering the difficulties involved, the volume concentrations calculated from axon diameters  $\rho_n$  and those obtained from the low frequency resistances by equation (1) assuming a non-conducting membrane,  $\rho$ , agree rather well. There is some spread in the data, and if we take the maximum deviation we obtain, at least as a lower limit, a membrane resistance of 3 ohm cm<sup>2</sup> from equation (4).

Five satisfactory experiments were obtained in which an impedance locus could be plotted on the same axon both before and after loss of

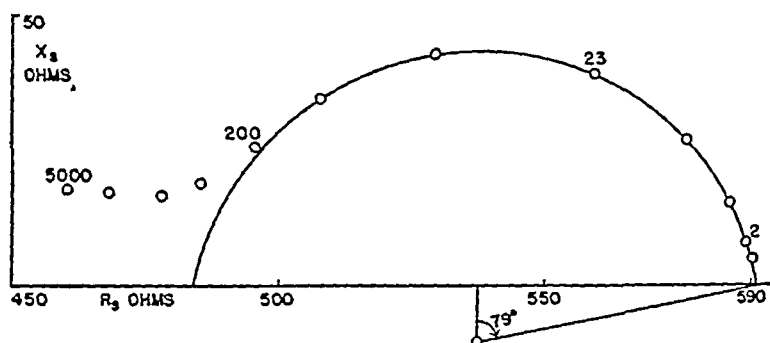


FIG. 2 Impedance locus, series resistance  $R_s$  vs series reactance  $X_s$ , for a single axon at room temperature. Frequencies given are in kilocycles per second.

excitability. These axons covered a considerable range of sizes and phase angles. The constants  $\phi$ ,  $c_M$ ,  $r_2$ , and  $r_3$  were computed for each axon in the two conditions and the average change of each constant was negligible.

As has been mentioned, several hours after the axons lost excitability, the low frequency resistance and capacity would start to decrease until the membrane capacity became zero and the internal resistance was that of sea water.

#### DISCUSSION AND CONCLUSIONS

It is unfortunate that the data are not more consistent and reproducible from one axon to another. But the axons are delicate and are not excitable unless they have been handled quite carefully, so it may be that the results are as good as should be expected. There are



indications that the phase angle of the *Arbacia* egg membrane may be affected by rough handling (Cole and Spencer, 1938, Cole and Curtis, 1938) But since the phase angle and the other constants of the axons do not change on loss of excitability, we hardly feel justified in explaining the wide variability in the excitable axons as due to injuries which were not sufficient to prevent excitability Transverse impedance measurements must by their very nature be made in a higher and more difficult frequency range from the longitudinal measurements and furthermore, the extra resistance incurred by the use of large electrodes alone increases the necessary precision of the measurements tenfold

In spite of the variations, it seems reasonably certain that over a large frequency range, the squid giant axon membrane has a polarization impedance with a phase angle of about  $76^\circ$  and a capacity at 1 kc of about  $1 \mu\text{f}/\text{cm}^2$  Although it was suggested (Cole and Curtis, 1936) that the whole nerve data could be interpreted on the basis of a statistical distribution of diameters and static membrane capacities among the axons, further calculations made this assumption seem rather improbable, particularly in view of the fact that a polarization impedance has been found for the single cell *Nitella* Consequently the polarization impedance is not particularly surprising, nor is the average axon membrane capacity entirely unexpected, in view of the wide variety of cell membranes which have capacities of the order of one  $\mu\text{f}/\text{cm}^2$

A few measurements were made on the fin nerves of the squid, with many small axons covering a wide range of diameters, which gave phase angles between  $40^\circ$  and  $50^\circ$  This is a much lower value than has been found for any single cells or reasonably uniform material We may assume that it is due in part to phase angles less than  $90^\circ$  for the individual membranes, and in part to the distribution of diameters A similar explanation can probably be given for data on other whole nerves

If then a polarization impedance can be an inherent characteristic of a single cell membrane, the problem of the membrane structure is somewhat clarified Although, as has been said, there is no adequate picture of this polarization mechanism, we may now look upon it as a general problem, of which the static capacity is a special case

Perhaps the most interesting observation is that the impedance

properties of the axon did not change when it became inexcitable. It has been observed that the resting potential does not alter markedly under the same conditions, but it seems difficult to align these two types of measurements at present. The constancy of the red blood cell membrane capacity in chemical hemolysis (Fricke and Curtis, 1935) is probably more nearly analogous. Since the phase angle and capacity are undoubtedly determined by the membrane structure and composition, we are led to assume that the changes accompanying hemolysis and loss of excitability are either slight, if the membrane is homogeneous, or else take place in only a small fractional part of the area if the membrane is not uniform. It must be remembered that suspension and transverse measurements are not suitable for the determination of the resistance, or change of resistance, of the membrane to direct current because by these methods, it is not yet possible to measure or even detect a conductivity when the resistance is above  $100 \text{ ohm cm}^2$ . The *Nitella* membrane has a resistance of  $100,000 \text{ ohm cm}^2$  or more (Blinks, 1930) and preliminary measurements have indicated that the normal axon membrane resistance may be of the same order of magnitude.

It might well be that excitability would be lost when this resistance fell to  $1000 \text{ ohm cm}^2$ , but it does seem unreasonable to suppose that so large a change would leave the membrane phase angle and capacity unchanged if it took place over the entire membrane surface. If then, after loss of excitability, the resistance continued to decrease, more and more current would flow through the membrane and cell interior until it would become measurable at a membrane resistance of about  $1 \text{ ohm cm}^2$ . If it were then to go as low as  $0.01 \text{ ohm cm}^2$  it would be very difficult to detect any evidence of a normal membrane polarization impedance, and as yet we have no way to determine whether the final drop in the low frequency parallel resistance and capacity is due to a disintegration of the membrane structure responsible for the polarization impedance or whether it remains essentially intact and the current flow is increasingly diverted as highly localized areas have less and less resistance.

There is not sufficient information available at present to determine the nature of the effects which were observed in the excitability tests. The preliminary humps on the cathode ray figure may be explained

as either a change of impedance or an unequalized potential. Also, it is not possible to locate and explain the origin of the action potential satisfactorily for the electrode arrangement used.

#### SUMMARY

The impedance of the excised giant axon from hindmost stellar nerve of *Loligo pealii* has been measured over the frequency range from 1 to 2500 kilocycles per second. The measurements have been made with the current flow perpendicular to the axis of the axon to permit a relatively simple analysis of the data. It has been found that the axon membrane has a polarization impedance with an average phase angle of  $76^\circ$  and an average capacity of  $1 \mu\text{f}/\text{cm}^2$  at 1 kilocycle. The direct current resistance of the membrane could not be measured, but was greater than  $3 \text{ ohm cm}^2$  and the average internal specific resistance was four times that of sea water. There was no detectable change in the membrane impedance when the axon lost excitability, but some time later it decreased to zero.

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## THE ACCUMULATION OF ELECTROLYTES

### XI ACCUMULATION OF NITRATE BY VALONIA AND HALICYSTIS

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In the course of preliminary experiments on the penetration of nitrates into *Valonia macrophysa*, Kütz, in Bermuda in the winter of 1935-36, it was found that living cells invariably contained significant concentrations of nitrate ions

Previously Wodehouse<sup>1</sup> had shown that *Valonia* sap gave a test for nitrate by means of the nitron reagent, although the surrounding sea water did not. An analysis made in this laboratory on preserved sap<sup>2</sup> yielded the low value of 0.002 M, and it was conjectured that it might be higher in fresh sap. In 1934, Ullrich,<sup>3</sup> working in Naples, found that freshly collected cells had no nitrate detectable by the phenol disulfonic method but that after 5 days in the circulating sea water of the station the nitrate concentration rose to the neighborhood of 0.015 M, which was also the average concentration of nitrate in the circulating sea water at the time of the experiments. He did not find accumulation of nitrate in any of his experiments.

On the basis of earlier work we had assumed that the nitrate concentration of the sap could be neglected. The object of the present paper is to show that in *Valonia macrophysa* in Bermuda nitrate is accumulated to a large extent, as Wodehouse has already shown qualitatively.

Analyses made on *Halicystis Osterhoutii*, Blinks and Blinks in 1936-37 showed nitrate inside greater than outside by 500 times or more.

The work on *Valonia* falls into two parts, 13 analyses made in the

<sup>1</sup> Wodehouse R. P., *J. Biol. Chem.* 1917, 29, 453

<sup>2</sup> Osterhout W. J. V., *J. Gen. Physiol.*, 1922-23 5, 225

<sup>3</sup> Ullrich, H., *Planta*, 1934, 23, 146

winter of 1936-37 on the sap of cells which had been exposed to the circulating salt water system of the Bermuda Biological Station for varying periods from a few hours up to 6 weeks, and 10 analyses made in the winter of 1937-38 on sap extracted in the field immediately after collection

### *Analyses*

The presence of nitrate in the sap and in the sea water was demonstrated qualitatively by the method of Atkins,<sup>4</sup> using diphenylbenzidine, and in the case of sea water this method was used for the quantitative analysis. Because of the large amount of nitrate present it was not suitable for the sap and a method based on the use of Devarda's alloy, which reduces nitrate and nitrite to ammonia, was substituted.

The analysis was carried out as follows. 0.1 ml of centrifugalized sap was transferred to the distillation flask of a micro-ammonia steam distillation apparatus similar to that described by Teorell.<sup>5</sup> 5 mg of Devarda's<sup>6</sup> alloy was then added and the flask was connected to the apparatus. Then 2 ml of 30 per cent sodium hydroxide was admitted to the flask and reduction started by applying a small flame for a few moments. The escaping hydrogen was made to pass through the condenser, through 10 ml of 0.05 N sulfuric acid, and finally into the air through a trap consisting of glass pearls wet with 0.05 N sulfuric acid. In this way loss of ammonia during the reduction stage was avoided. After 10 minutes reduction, the ammonia was distilled in the steam current into the sulfuric acid and its quantity was determined by Nessler's reagent, using the Zeiss-Pulfrich step-photometer as described in a previous paper.<sup>7</sup>

A determination of ammonia was made on each sap sample by steam distillation without the addition of the reducing alloy, and the result of this was subtracted from the total nitrogen found by the first procedure, to give nitrate + nitrite nitrogen.

Several samples of sap also were tested for nitrite, using the dimethyl aniline hydrochloride test. These invariably gave less than 0.0001 M nitrite nitrogen which was about the lower limit of sensitivity as we used the method. Nitrite nitrogen may therefore be neglected.

The diphenylamine test for nitrate is, as Ellert<sup>8</sup> has pointed out, also given

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<sup>4</sup> Atkins, W. R. G., *J. Marine Biol. Assn. United Kingdom*, Plymouth, 1932, 18, 167.

<sup>5</sup> Teorell, T., *Biochem. Z.*, Berlin, 1932, 248, 246.

<sup>6</sup> Devarda, A., *Chem. Zentr.*, 1897, 2, 64.

<sup>7</sup> Jacques, A. G., *J. Gen. Physiol.*, 1937-38, 21, 665.

<sup>8</sup> Ellert, L., *Pharm. Zentralhalle*, 1925, 66, 649 (quoted from *Chem. Abstr.*, 1926, 20, 158).

by iodates, chlorates, perchlorates bromates, chromates, and bichromates, tungstates, molybdates, vanadates, ferricyanates and peroxides Atkins points out that the same substances must also give the diphenylbenzidine test He adds to the list arsenate and ferric iron Of these substances, the heavy metal acids are most unlikely to be present in the sap in detectable amounts Ferric iron has been found to be absent by testing with thiocyanate Iodate and bromate have been excluded by adding KI to the sap in the presence of dilute sulfuric acid and chloroform If either iodate or bromate had been present in appreciable amounts the iodine produced would have colored the chloroform This test is better for iodate than for bromate, but the absence of an important amount of the latter is also proved Chlorate and bromate were tested for by the sea water method of Korenman,<sup>9</sup> and found to be absent Perchlorate is scarcely likely to be present in any important amount and this is true also for peroxides In any case the absence of these latter was proved by a test with 'luminol,' or 3 amino phthalhydrazide (not 'luminol'), by a method suggested to the writer by O Baudisch<sup>10</sup>

It seems clear therefore that the strong diphenylbenzidine test we obtained is valid evidence of a considerable amount of nitrate in the sap

The analyses however were made by reducing the nitrate and determining ammonia It is therefore necessary to consider how the results may be affected by other sources of ammonia Brandt<sup>11</sup> used aluminum amalgam in a solution made alkaline by magnesium oxide to reduce nitrate and nitrite, but his method has been criticized by Harvey<sup>12</sup> for the very small amounts of nitrate in the sea water on the ground that the treatment may decompose organic nitrogen compounds such as amino acids and proteins His criticisms were applied to the case of sea water They can scarcely apply to our analyses of the sap where the nitrate content is at least several thousand times greater The sap contains too little protein even if it could be all decomposed by reduction, so as to give its nitrogen in the form of ammonia, to affect our results seriously and the absence of all but a doubtful faint trace of amino acid has been established by testing the sap according to the method of Folin<sup>13</sup> We may say therefore with considerable confidence that our method gives reasonably accurate values for nitrate in the sap

This applies chiefly to *Valonia* *Halicystis* sap has not been studied so much

<sup>9</sup> Korenman, I M, *Mikrochemie* 1936, 20, 225

<sup>10</sup> According to this method a dilute alkaline solution of 3 amino phthalhydrazide is treated with a few drops of a dilute hemin solution, and to this is added a trace of the test solution In the presence of peroxides the mixture luminesces strongly *Valonia* sap failed to excite the luminescence, but a drop of a very dilute  $H_2O_2$  solution did so

<sup>11</sup> Brandt K., *Nova acta Abh d k Leop Carol Deutsch Akad Naturf*, Halle, 1915 Vol C No 4

<sup>12</sup> Harvey, H. W, *Rapp conseil perm internat expl mer*, 1929, 53, 72

<sup>13</sup> Folin, O, *J Biol Chem*, 1922, 61, 377

and moreover the sap used here contained much larger, and more variable, amounts of ammonia, as determined by distillation from alkaline solution and Nesslerization. This is in distinct contrast with the previous analyses of *Halocystis* sap<sup>14</sup> which contained no appreciable amount of ammonia. Blinks<sup>15</sup> has suggested that the difference in ammonia content of *Halocystis* cells probably depends on age and condition. Old cells, such as were used in the present case, which have gone through several reproductive periods, invariably contain considerable quantities of dark deposits in the sap and it is presumed that the decomposition of this material furnishes ammonia, since cells with the dark material always have ammonia in them. Before analysis the sap was centrifugalized, but there remains the possi-

TABLE I  
*Analyses of Sap of Valonia for Nitrate*

Description of locality	Concentration nitrate + nitrite* in sap	Concentration nitrate + nitrite in sea water	Ratio $\frac{(\text{NO}_3)_{\text{sap}}}{(\text{NO}_3)_{\text{s.w}}}$
	M	M	
Shallow lagoon with circulation for about 12 hours a day	0.0022	$5 \times 10^{-6}$	440
Shallow tidal pool sheltered from the sun	0.0134	$7 \times 10^{-7}$	19143
Deep pond with slow circulation	0.0242	$9 \times 10^{-6}$	2690
Deep pond with slow circulation	0.0221	$5 \times 10^{-6}$	4420
Tidal inlet	0.0187	$1 \times 10^{-5}$	1870
Tidal shallow cove	0.0377	$1 \times 10^{-5}$	3770
Open shore	0.0300	$1 \times 10^{-5}$	3000
Artificial ponds at head of tidal inlet	0.0172	$1 \times 10^{-5}$	1720
Tidal pool	0.0113	$9 \times 10^{-6}$	1256
Tidal pool	0.0115	$9 \times 10^{-6}$	1277
Station circulating salt water system (3 days)	0.0111	$5 \times 10^{-6}$	2220
Average	0.0181	$7.5 \times 10^{-6}$	2387
Average deviation from the mean	0.0077		

\* Nitrite was shown to be negligibly small in both sap and sea water

bility that soluble nitrogenous substances, arising from the decomposition of the insoluble organic material, may have given rise to ammonia on reduction. However, it seems probable that there is some nitrate in normal *Halocystis* sap, although the amount is smaller than in sap of *Valonia*.

In the first stage of the work (season 1936-37) the average concentration of nitrate in *Valonia* sap was found to be 0.0143 M, and the

<sup>14</sup> Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929-30, 13, 733

<sup>15</sup> Blinks, L. R., *J. Gen. Physiol.*, 1933-34, 17, 109. Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, 1931, 57, 389



average deviation from the mean 0.0023 M (13 samples) For *Halcystis* the average concentration of nitrate was 0.0043 M and the average deviation from the mean 0.0019 M (3 samples)

Each group of *Valonia* cells taken for analysis was different in its history but this seemed to make no great difference in the nitrate content In all cases, however, the cells were exposed to the circulating salt water of the Biological Station However, tests of the circulating sea water at various times gave only faint traces of nitrate But in view of Ullrich's results which came to our attention after the completion of the first part of the work it seemed desirable to test sap samples expressed in the field and to compare the nitrate content with that of the sea water collected in the vicinity of the cells at the time the sap samples were expressed The results of these analyses made in the winter of 1937-38 are given in Table I

#### DISCUSSION OF RESULTS

As Table I shows the nitrate content of the sap was found to be in all cases much greater than that of the sea water surrounding the cells, the average accumulation in *Valonia* being more than 2000 fold Moreover, it is not impossible that the nitrate values for the sea water may be too high, especially in view of the statement of Rakestraw<sup>16</sup> that "The vicinity of Bermuda is an area especially devoid of nitrite and other 'nutrients' A number of surface samples were collected from the harbors and bays of the Bermuda Islands, and even from their fresh water streams and ponds, with no more than traces of nitrite, and minimal amounts of nitrates" As pointed out above the diphenylbenzidine test for nitrate is given by other oxidants and these may have been present in the sea water samples which were necessarily taken along the shore at low tide, when the cells are most easily collected Under these conditions it would not be surprising if the sea water were modified considerably by metabolism

The minimum amount of accumulation in *Valonia* is therefore more than 2000 fold

This is of interest in view of a recent statement that it is doubtful whether *Valonia* can accumulate any other anion than chloride<sup>17</sup>

<sup>16</sup> Rakestraw, N W *Biol Bull*, 1936, 71, 133

<sup>17</sup> Steward, F C, and Martin, J C, *Carnegie I* No 475, 1937, 87

In considering the possible sources of energy responsible for the accumulations, there is apparently no nitrate with a gradient of chemical potential in the required direction. The manner in which the energy necessary for accumulation is applied is not clear, but a number of possibilities suggest themselves. (1) That nitrogen or nitrogenous compounds in the sea water, such as proteins, nitrites, or ammonia pass into the sap and are oxidized in the cell to nitrates.<sup>18</sup> (2) That the cell itself or bacteria found on the surface of the cell utilize nitrogen to form soluble nitrogenous compounds, and these pass into the cell and are there oxidized to nitrates,<sup>19</sup> and (3) that nitrate is formed by the cell itself or by bacteria at the external surface of the protoplasm. If bacteria manufacture the nitrate they supply the chemical energy and the nitrates pass into the cells by means of diffusional energy. In all other cases the cell itself supplies at least a part of the energy in the form of chemical energy.

It is clear that if the nitrate is manufactured inside the cell, the exit of nitrate must be very slow. The evidence in this respect is contradictory. Thus the results of Ullrich<sup>3</sup> described above suggest that nitrate passes freely in and out of the cell and comes rapidly into equilibrium in the sap and sea water. On the other hand we find that cells which have been kept as long as 3 months at 15°C in dim light in sea water practically free from nitrate still contain appreciable amounts of nitrate. Moreover there is no evidence that the sea water in contact with them has gained nitrate. Under these circumstances we may assume either that the exit is very slow or that the cell gradually consumes the nitrate. This must be left to future investigation.

<sup>18</sup> Moore, Whitley, and Webster (Moore, B., Whitley, E., and Webster, T. A., *Proc. Roy. Soc. London, Series B*, 1921, **92**, 51) believe that *Enteromorpha compressa* is able to synthesize soluble nitrogenous compounds from nitrogen dissolved in sea water.

<sup>19</sup> At the present time the weight of the evidence seems to be against the direct fixation of nitrogen by green algae. It is now supposed that the fixation takes place through bacteria which, according to a number of investigators, are regularly found on the surface of certain algae. In the case of *Valoniopsis macrophylla*, evidence of bacterial activity is found in the summer in Bermuda when cells are stored in still water. The cells and even the walls of the containing vessels become covered with thick layers of a jelly-like material which gives the cells a slippery feeling.

It should be pointed out, however, that (as shown in another paper<sup>20</sup>) when the nitrate content outside the cell is increased very greatly, its concentration inside also increases which indicates that nitrate can pass through the protoplasm

Almost invariably in our work we have found the total cation concentration, ( $K^+ + Na^+$ ) or in some cases ( $K^+ + Na^+ + NH_4^+$ ), to be greater than the halide concentration<sup>21</sup> On the basis of Van der Pyl's careful analysis Osterhout and Dorcas calculated the difference to be 1.9 per cent<sup>1</sup> They suggested that the discrepancy would be accounted for if nitrate, bicarbonate, and organic anions were taken into account From 25 analyses made at various times<sup>22</sup> on groups of cells under normal conditions the average concentration of ( $K^+ + Na^+$ ) was 0.6231 M and for halide 0.6134 M, a difference of 0.0097 or 1.6 per cent This discrepancy is more than covered by the concentration of nitrate in the sap for which an average<sup>23</sup> value of 0.0161 M has been obtained The discrepancy is now 1.0 per cent in the other direction, i.e., we have an excess of halide plus nitrate This is perhaps due to small amounts of calcium, magnesium, and ammonia in the sap which are not taken into account

Our results differ from those of Stewart and Martin<sup>17</sup> who state that in *Valonia macrophysa* of Tortugas the halide concentration is almost always greater than ( $K + Na$ ) and that for the average of 29 analyses ( $K^+ + Na^+$ ) = 0.620 M and halide = 0.624 M so that the excess halide = 0.64 per cent

#### SUMMARY

The nitrate concentration in the sap of *Valonia macrophysa*, Kütz., is at least 2000 times that of the sea water, and in *Halocystis Osterhoutii*, Blinks and Blinks, at least 500 times that of the sea water

<sup>20</sup> Jacques, A. G. *J. Gen. Physiol.*, 1937-38, 21, 775

<sup>21</sup> Jacques, A. G., and Osterhout, W. J. V. *J. Gen. Physiol.*, 1930-31, 14, 301, 1931-32, 15, 537 1933-34, 17, 727 Osterhout, W. J. V. and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, 7, 633

<sup>22</sup> Jacques, A. G. and Osterhout, W. J. V. *J. Gen. Physiol.*, 1930-31, 14, 301 1931-32, 15, 537 1933-34, 17, 727 Also unpublished results

<sup>23</sup> This is the average of all 24 analyses made in 1936-37 and 1937-38



## THE ACCUMULATION OF ELECTROLYTES

### VII ACCUMULATION OF HALIDE AND NITRATE BY VALONIA IN HYPERTONIC SOLUTIONS

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The ratio of halide to nitrate in Bermuda sea water, as shown in a previous paper,<sup>1</sup> is at least 80,000 to 1,<sup>2</sup> and in the sap the average value is about 38 to 1. On this basis we might conclude that the nitrate penetrates much more rapidly than the halide unless the nitrate is formed in the cell from other nitrogen compounds<sup>3</sup> or from atmospheric nitrogen.

If we decrease the ratio of halide to nitrate in the sea water, we might expect a corresponding change in the sap. This occurs but the change is smaller than was expected, and it is independent of the extent of the change of the ratio of halide to nitrate in the sea water.

By increasing the osmotic pressure of the sea water the cells can

<sup>1</sup> Jacques A G, *J Gen Physiol*, 1937-38, 21, 767

This value has been calculated from the analyses of Bermuda sea water samples collected in the localities where *Valonia* occurs. But, as pointed out in the previous paper (footnote 1), the figures obtained may be much too high, since the method of analysis by diphenylbenzidine is not specific for nitrates but includes many other oxidants which are possibly present in sea water along the shore. In this connection Rakestraw (Rakestraw, N W, *Biol Bull*, 1936 71, 133) has stated that the surface water in the vicinity of Bermuda is exceptionally low in both nitrites and nitrates. In any case it is clear that there is a remarkable accumulation of nitrate in the sap.

<sup>2</sup> Moore, Whitley, and Webster (Moore B, Whitley, E, and Webster T A, *Proc Roy Soc London Series B* 1921, 92, 51) believe that some marine green algae are able to synthesize soluble nitrogen compounds from atmospheric nitrogen. Others say that this process is due to nitrogen fixing bacteria on the surface of the algae. If this process takes place the nitrate concentration of the sea water in contact with the cell surface may be much greater than that of the sea water as a whole.

be made to take in electrolytes even in dim light where the growth is negligible and this device has been resorted to in the present case. It has been found that the concentration of the sea water may be raised abruptly as much as 10 per cent without permanently injuring the cells or causing plasmolysis. The cells soften at first by losing water, but they rapidly regain their turgor.

TABLE I

*Entrance of Halide and Nitrate Into Valonia macrophysa from Hypertonic Sea Water with Varying Halide Concentrations*

Time	Halide concentration (M) in sap of cells in modified sea waters					Nitrate concentration (M) in sap of cells in modified sea waters				
	A	B	C	D	E	A	B	C	D	E
hrs										
0	0 6106 Av 0 6096 0 6068					0 0171 Av 0 016 0 0150				
84	0 6328	0 6321	0 6431	0 6366	0 6321	0 0232	0 0200	0 0193	0 0257	0 0285
156	0 6309	0 6378	0 6393	0 6366	0 6328	0 0254	0 0322	0 0286	0 0221	0 0300
180	0 6339	0 6481	0 6481	0 6366	0 6366	0 0392	0 0300	0 0254	0 0378	0 0342
276	0 6409	0 6500	0 6470	0 6546	0 6443	0 0346	0 0270	0 0390	0 0325	0 0257

Molar composition of sea water

	Halide	Nitrate	Halide + nitrate	Ratio halide/nitrate*
A	0 400	0 229	0 629	1 75
B	0 486	0 143	0 629	3 41
C	0 572	0 057	0 629	10 00
D	0 600	0 029	0 629	20 70
E	0 623	0 0057	0 6287	109 30

\* The ratio in ordinary sea water is at least 80,000 to 1, the concentration of halide being 0.58 M and of nitrate  $7.5 \times 10^{-6}$  M at most. Probably it is much less.

## EXPERIMENTAL

The sea waters were modified by adding 1.2 M  $\text{NaNO}_3$  solution, 1.2 M  $\text{NaCl}$  solution, and 0.6 M  $\text{NaNO}_3$  solution in such proportions as to produce the desired increase in the electrolyte concentration and decrease in the external ratio of  $\text{Cl}/\text{NO}_3$ .<sup>4</sup> The cells were then placed in the modified sea water in deep rectangu-

<sup>4</sup> This, of course, lowered the external concentration of calcium, magnesium, and sulfate.

lar covered Pyrex dishes, which were mechanically rocked very slowly. At intervals cells were withdrawn for analysis for halide and nitrate.

Halide was determined by titration with  $\text{AgNO}_3$ , the end point being found electrometrically, using a silver electrode.

Nitrate was determined by Devarda's method as described in another paper.<sup>1</sup> Ammonia was determined in each sample and a suitable correction applied to the nitrate determination. This correction was in general less than 2 per cent.

The results of a typical experiment are given in Table I. The experiments were carried out in Bermuda in the winter of 1935-36.

### DISCUSSION OF RESULTS

Because of natural variations in the halide and nitrate concentrations of the sap, the results are too irregular to permit a mathematical treatment of the kinetics. It is clear, however, that as a result of raising the osmotic pressure of the sea water in the manner described the concentrations of both halide and nitrate have increased in the sap. At the end of the experiment the concentration of halide + nitrate inside was greater than that outside by about 0.05 M which is approximately the same difference as in the cells in normal sea water.

When the external halide is greatly decreased, as in sea water A, we might expect halide to come out of the cell in exchange for incoming nitrate. Instead of this we see that halide continued to enter. Obviously then the ratio of halide to nitrate in the sap could decrease only if nitrate entered relatively faster than in ordinary sea water. This evidently took place. In ordinary sea water halide entered 38 times as fast as nitrate. In sea water A the increase in concentration of halide in the sap in 276 hours was 0.0313 M and of nitrate 0.0186 M, giving a ratio of  $0.0313 / 0.0186 = 1.68$ , not very different from the ratio 1.75 found in the external solution. We cannot make a more definite statement unless we know the actual concentration gradients.<sup>5</sup>

<sup>5</sup> If we assume for purposes of calculation, that chloride enters as  $\text{NaCl}$  and nitrate as  $\text{NaNO}_3$ , we have the following concentration gradients at the start in sea water A (we assume, as in previous papers, that the relative rates of entrance are proportional to the inwardly directed concentration gradients in the non-aqueous protoplasmic surface and that these in turn are proportional to the difference in ionic products in the external solution and in the sap: the subscripts  $o$  and  $i$  refer to outside and inside respectively),  $[\text{Na}_o][\text{Cl}_o] - [\text{Na}_i][\text{Cl}_i] = 0.5 (0.4)$

It is not surprising that the decrease of the ratio of halide to nitrate in the sap was gradual for if no halide comes out the decrease in ratio must be gradual when electrolytes enter as slowly as in this experiment. In sea water A the ratio of halide to nitrate in the sap decreased from 38 to 18.5 in 276 hours.

The fact that the increase in nitrate concentration in the sap has not kept pace with that in the sea water has caused the ratio of nitrate in the sap to that in the sea water to fall off. This ratio decreases as the outside concentration of nitrate increases, and becomes less than unity in sea waters A, B, and C. The question whether there is a relative change in the rates of entrance of nitrate and halide as the ratio of halide to nitrate in the sea water changes from 100 to 1.75 cannot be satisfactorily settled. Since the change of ratio in the sea water from 80,000 to 109.30 merely resulted in doubling the concentration of nitrate in the sap a further change to 1.75 could scarcely affect the result very greatly during the time of the experiment. The ratio of halide in sap to halide in sea water increased in all cases except in solution E, the greatest increase being in solution A, from 1.05 to 1.59.

Thus we seem to have an induced halide accumulation greater than that found under normal conditions. Since it is by no means certain that the cell has settled down to a steady state a further increase of concentration may be possible.

We assume that the movement of substances through the non-aqueous surface layer of the protoplasm is chiefly in molecular form. The following molecules are available for the transfer of chloride, NaCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, KCl, and HCl. The gradient of chemical potentials of the first three is directed inward, and of the last two outward. The net movement of chloride will depend on the flux of all these molecules.

On this basis it might be suggested that chloride enters the cell chiefly as NaCl.<sup>6</sup> If all the chloride has entered as NaCl, we should

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$-0.1(0.6) = 0.110$  and  $[Na_o][NO_{3o}] - [Na_i][NO_{3i}] = 0.5(0.23) - 0.1(0.016) = 0.113$ . Hence the concentration gradient of NaCl is about 25 per cent greater than that of NaNO<sub>3</sub>.

<sup>6</sup> As the sap and sea water contain much less magnesium and calcium than sodium, their role may be unimportant and the discussion will therefore be confined to NaCl.



expect to find as much sodium as chloride in the sap. But this is not necessary if sodium enters as NaCl and goes out in combination with another anion.<sup>7</sup>

In normal sea water the gradients of chemical potential of  $\text{NaNO}_3$ ,  $\text{KNO}_3$ , and  $\text{HNO}_3$  are directed outward. The status of  $\text{Mg}(\text{NO}_3)_2$  and of  $\text{Ca}(\text{NO}_3)_2$  is not so clear. If the average nitrate concentration of the sap is 0.016 M and that of the sea water is taken as  $7.5 \times 10^{-6}$  M the concentration of magnesium in the sap would have to be only  $1.3 \times 10^{-8}$  M to make the gradient of chemical potential for  $\text{Mg}(\text{NO}_3)_2$  zero. To make the gradient of chemical potential of  $\text{Ca}(\text{NO}_3)_2$  zero the concentration of calcium in the sap would have to be slightly less than  $2.6 \times 10^{-9}$  M.<sup>8</sup> According to the results of Steward and Martin<sup>9</sup> far more than the required amounts of calcium and magnesium are present in cells at Tortugas.

It might be assumed that halide and nitrate enter by exchange with bicarbonate ion. It is possible that when the concentration of electrolytes in the sea water is increased, the entrance of both potassium and sodium increases. As a result the rate of exchange of bicarbonate ions for halide ions might increase, since the entrance of the bases would increase the pH of the sap (particularly in the layer next to the protoplasm) and therefore the concentration of the bicarbonate ion. Under these conditions it would be the concentration of the relatively scarce bicarbonate ion and not of the abundant halide ion which would determine the rate of entrance of halide. But if the surface of the protoplasm is a non aqueous liquid of low dielectric constant, as potential measurements suggest, the rôle played by ionic transport is probably very small.

It is evident that the mechanism of accumulation of anions in these experiments needs further investigation.

<sup>7</sup> This will be discussed in a later paper.

<sup>8</sup> These results are based on the analyses of sea water as given by Osterhout (Osterhout, W. J. V. *Ergebn. Physiol.*, 1933, 35, 967). The assumption is made that the activity coefficient for Ca is the same in the sap and sea water. The same is true for magnesium and for nitrate. Osterhout's sap analyses show much more than the required amount of calcium and a trace of magnesium which might easily be as much as  $1.3 \times 10^{-8}$  M.

<sup>9</sup> Steward, F. C., and Martin, J. C. *Carnegie Institution of Washington Pub. No. 475*, 1937, 87.

## SUMMARY

When cells of *Valonia macrophysa* were placed in hypertonic sea water, the concentration of halide and of nitrate increased, and the sum of halide + nitrate became 0.05 M greater inside than outside, which is about the same difference as is found in cells in normal sea water.

In ordinary sea water the ratio of halide to nitrate is 80,000 to 1. When this was changed by substituting nitrate for halide so that the concentration of halide was 1.75 times that of nitrate the rate of entrance of halide was 1.68 times that of nitrate in 276 hours and the ratio of halide to nitrate in the sap decreased from 38 to 18.5.

No halide came out in exchange for entering nitrate. The retention of chloride may well be due to the fact that even when the halide concentration of the sea water is reduced as low as 0.4 M, there is still an inwardly directed activity gradient of sodium chloride.

## EXCITATION OF NERVE FIBERS IN THE SQUID (*LOLIGO PEALII*)

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(Accepted for publication, April 18, 1938)

In the nerves of the squid axons with diameters up to nearly 1 mm provide a unique opportunity for the study of the excitation of so called non medullated nerves. Of special interest is the opportunity to find out whether the excitation is the same in an isolated nerve fiber as when that fiber is surrounded by other fibers in a nerve trunk.

Excitation in single nerve fibers has been studied in medullated nerve with the aid of pore electrodes (Grundfest, 1932, Sakamoto, 1933), by stimulating so as to excite only the most excitable fiber in a group (Blair and Erlanger, 1936), and by the isolation of one nerve fiber (Kato, 1934). These studies have shown that the time constants of excitation of single fibers in frog motor nerves are approximately the same as when a large group of fibers are stimulated. It has not previously been possible, however, to measure the excitation properties of a given nerve fiber both isolated and intact in a trunk.

The large fibers in the stellar nerves of the squid (Young, 1936 *a* and *b*, Bear, Schmitt, and Young, 1937), innervate the circular muscle fibers of the mantle. These nerve fibers range from 100 to 800 micra in diameter. A single condenser discharge, provided it is not too long or too strong, sets up a single impulse in the giant nerve fiber, this impulse then causing contraction of all the muscle fibers reached by the axon (Young, 1938). There are also smaller fibers (up to 50  $\mu$  in diameter) in the stellar nerves and some of these are motor axons to circular muscle fibers. It is possible to dissect away the giant fiber completely from all surrounding fibers and, since its

\* The authors are indebted to John Z. Young of Oxford University for assistance in making the preparations in many of the early experiments, also for helpful criticism of the manuscript.

threshold is nearly always lower than that of the smaller fibers, it is also possible to stimulate it independently without isolation. Since the tension produced through the smaller nerve fibers is at most only a very small fraction of that produced by the giant fiber, it is certain that when a large contraction of the muscle occurs the giant fiber has been stimulated. When the giant fiber is stimulated repetitively the muscle shows no increase in tension as the frequency of stimulation increases above fusion frequency (Prosser and Young, 1937).

For study of the excitability of smaller axons it is convenient to use the fin nerve, which contains fibers up to  $50\ \mu$  in diameter, the response to whose stimulation is a contraction of the muscles of the fin.

Preparations were made as described by Young (1938), the stellate ganglion and its nerves being exposed by a median ventral cut through the mantle. Of the ten or so stellar nerves the hindmost ("great stellar nerve") is much the longest, and contains the largest giant fiber. It is the most convenient to prepare and has been used in most of the following experiments, the smaller fibers in the more anterior nerves being studied occasionally for comparison. The nerve was tied close to the ganglion, freed from surrounding tissues, and placed on electrodes. The muscle and nerve were bathed with sea water. Most of the experiments were at  $22\text{--}23^\circ\text{C}$ . The muscle nerve preparation is not very viable and rarely remained in good condition for more than 20 minutes, as judged by constancy of threshold and size of response.

In the majority of the experiments the stimulating electrodes were calomel cells. These were made up with sea water and their ends dipped into paraffin tubes 6 cm long leading to troughs in which were strips of wood 0.5 mm in diameter. The tubes and troughs in the paraffin chamber were filled with sea water. The nerve lay across the edges of the sea water-soaked pieces of wood.

The D.C. resistance of the electrodes was 4000 ohms. The resistance of 10 mm of isolated giant fiber was approximately 25,000 to 32,000, and of 2 mm was approximately 5000–10,000 ohms. The resistance of the entire nerve was considerably less.

A few experiments were performed with chlorided silver wire electrodes, a few with chlorided silver plates, and a very few with agar-sea water pore electrodes into which dipped coils of chlorided silver wire.

Stimulation was by single condenser discharges. The circuit used was similar to that of Hill (1934), but a hand switch was used in place of the commutator. Either 50,000 or 100,000 ohms were in series with the tissue, and this resistance was shunted by 500 to 10,000 ohms. The time to discharge to  $1/e$  of the charging potential was taken as the product of the condenser capacity and the shunt resistance ( $RC$ ).<sup>1</sup>

<sup>1</sup> The authors are indebted to Dr. D. Y. Solandt for the loan of some of the apparatus used in these experiments.

*Methods of Treating Experimental Data*

The data from many experiments were first plotted in the conventional manner of voltage ( $V$ ) against duration ( $RC$ ). Chronaxie values ( $0.37 RC$  for  $V = 2 \times$  rheobase) were obtained directly from these strength duration curves.

A more useful method of plotting, however, is that which has been extensively used by Hill, Rushton, and their associates, namely  $\log V$  (or  $E$ ) against  $\log RC$ . Hill has shown (1936) that many data when plotted in this way fall along a curve described by the equation

$$\frac{E}{E_0} = \left( \frac{RC}{K} \right)^{\frac{1}{K} - 1}$$

In the bi log plots of Figs 1 to 7, the line drawn in is the plot of this equation, the points were obtained experimentally. It will be seen from these figures that the fit is fairly good, in general it is better for the giant fibers intact in the great stellar nerve than when isolated (e.g. Fig 2). Characteristic times (Hill's  $K$  values) were read directly from these log log curves as the point where the slope of the tangent is  $-\frac{1}{2}$ . These points are indicated by arrows in the plots.

Blair has developed an equation which is similar to Hill's (Blair, 1935). It has the advantage of being tested by fit to a straight line by plotting  $\log RC \frac{E}{E_0}$  against  $\log RC + \log \frac{E}{E_0}$ . A number of our experiments were tested by this method of plotting, e.g., Figs 1 and 7. The fit is fair in many of them for durations shorter than chronaxie. Blair's  $K$  is obtained from the slope and intercept.

Crozier (1937) has shown that probability considerations may apply to the strength duration relation. He has applied the probability integral by plotting the excitability as a percentage of a saturation value, i.e.,  $100 \times \frac{E_0}{E}$  against  $\log RC$ . When plotted on probability paper a straight line should result if the probability integral applies. Data from a number of these experiments have been plotted in this way, e.g., Figs 1, 2, and 7. The fit is as good as with the equations of Hill and Blair. The 50 per cent point, inflection point, or mode, and the slope give constants which are comparable to time constants derived by other means.

It is clear from the figures of this paper and from many others which have been constructed that each of these three expressions describes the strength-duration curves extremely well. It is not the purpose of this paper, nor is it possible from these data, to decide whether any one of these equations fits the data better than the others. In any case all of these formulations are based upon the same general type of assumptions about the process of excitation. The mere fit of a given equation does not tell much about the mechanism of excitation and the value of each of these equations will come only insofar as experimentally testable physical meaning is given to them.

A few measurements of "accommodation" in the giant fiber have been made by Miss M. H. Huntington according to the method of Solandt (1936). These show that accommodation is slow in this fiber and  $\lambda$  values of 200–400 msec were obtained.

*Effect of Other Fibers in a Nerve Trunk upon the Excitation of One Nerve Fiber*

Strength-duration curves were obtained from the excitation of the giant fiber intact in the great stellar nerve in nine preparations. Calomel electrodes making contact through pieces of wood 10 to 15 mm apart were used. The characteristic times (Hill's  $K$ , or point where slope is  $-\frac{1}{2}$  on bi-log plot) range from 0.33 to 0.46 and average 0.42 msec. In fifteen preparations the giant fibers were isolated for approximately 2 cm. The piece of wood at the cathode made contact with the proximal portion of this isolated fiber. In some experiments the anode also was on the giant fiber only, in others it made contact with the entire distal end of the cut nerve. One of these experiments with an isolated giant fiber is presented in Fig. 1. With electrode separations of 10 to 15 mm the characteristic times of these isolated fibers ranged from 0.33 to 0.60 msec and averaged 0.47. These experiments show that the excitation time constants—characteristic times, chronaxies, Blair's  $K$ , and other similar constants—are essentially the same whether the fibers are intact in the nerve trunk or are isolated.

Fig. 2 gives the results of one of the experiments in which a strength-duration curve was obtained before and after isolation of a single

giant fiber This figure and the curves of other similar experiments show that the effect of the other nerve fibers and surrounding peri-

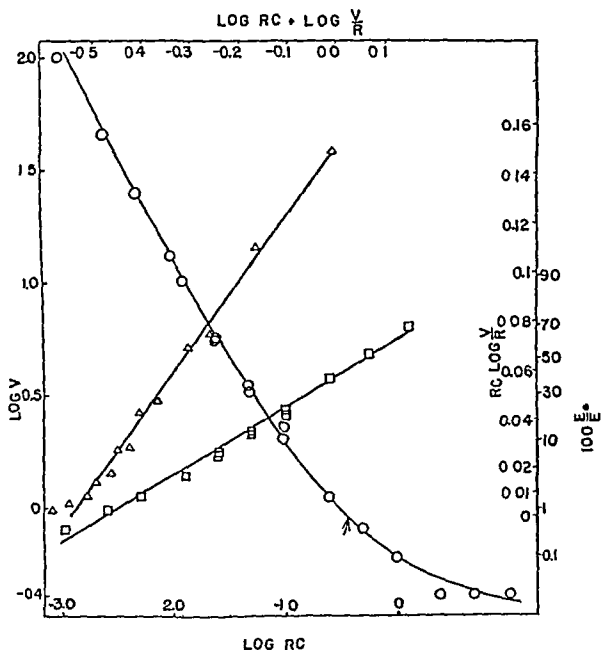


FIG 1 Strength-duration curve (circles) of an isolated giant fiber Calomel electrodes electrode separation 13 mm Solid curve—plot of Hill's theoretical equation, points obtained experimentally Arrow gives Hill's  $K$  value Lower straight line (squares)—probability plot of same data by Crozier's method ordinates at extreme right Upper straight line (triangles)—plot of same data according to Blair's equation, units on upper abscissae and inner right ordinates

neurium upon the excitation of one fiber is to raise the entire strength duration curve vertically The time constants remain

essentially the same. The same elevation of the curve can be obtained by laying a thread soaked with sea water along the isolated nerve fiber. When plotted according to the probability relation of Crozier, the points from the data of the isolated fiber fall along the same line as those from the fiber intact in the nerve trunk (Fig 2)

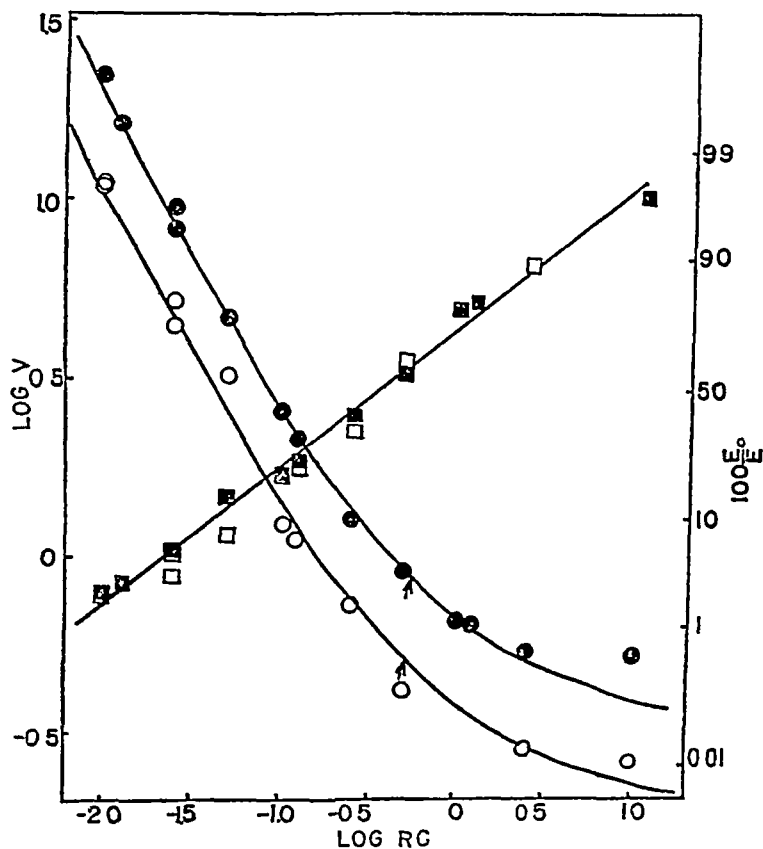


FIG 2 Strength-duration curve of same giant fiber when isolated (open circles) and when intact in great stellar nerve (solid circles). Straight line—probability plot of same data, solid squares—fiber intact in nerve, open squares—isolated fiber. Electrode separation 13 mm. Calomel electrodes.

This is understandable if the excitability as plotted in the log probability relationship does not change. Furthermore, since the resistance of the isolated fiber is higher than that of the whole trunk, the effect of the other fibers must be that of a shunt rather than to keep the current flowing through the preparation constant.

We conclude that the other fibers in a nerve, when the most sen-



sitive fiber is being stimulated at threshold, tend to shunt the stimulating current and thus to raise the voltage necessary to excite at any given duration. It is reasonable to assume that this holds for all fibers whether large or small. These experiments prove conclusively, therefore, that the shape of the strength duration curve and the time constants of a given fiber or group of fibers in a nerve trunk are the same as if that fiber or those fibers were isolated. The position of the strength-duration curve on the voltage axis is, however, higher for the nerve trunk.

### *Effect of Deterioration of the Preparation*

The exposed giant fiber and mantle is very sensitive to fatigue and to external conditions. This makes the preparation a very poor one for extended experiments. In fact it is often difficult to obtain sufficient points for a satisfactory curve. It has been found from action current recording (Young and Hartline, private communication) that the nerve often lasts much longer than the muscle. The muscular response may gradually weaken before disappearing. More often it ceases very abruptly, in such cases we believe that the neuromuscular junctions have broken down. In some few experiments the nerve showed a decrease in its excitability during a few minutes preceding the cessation of the response. One such experiment is shown in Fig 3. One curve is drawn through the points taken early in the experiment and the same curve is shifted to fit the points obtained later. The numbers give the order in which points were obtained. This experiment shows that with deterioration the entire curve is shifted diagonally upward and to the left with the result that the excitation time constants are shortened. In this experiment the  $K$  value in the fresh preparation is 0.58 msec, while when aged it is 0.47 msec. In the probability plots the slope is less steep as deterioration proceeds.

### *Effect of Interelectrode Distance*

Fig 4 shows strength duration curves for an isolated giant fiber at electrode separations of 9.5 and 1.5 mm. Fig 5 gives similar data for a fiber intact in the stellar nerve. The effect shown by these and other experiments is that as the interelectrode distance is decreased

the entire strength-duration curve is shifted diagonally upward and to the left. The excitation constants (chronaxie, characteristic time, etc) are shortened as a result. This effect has been observed by numerous investigators working with nerve trunks

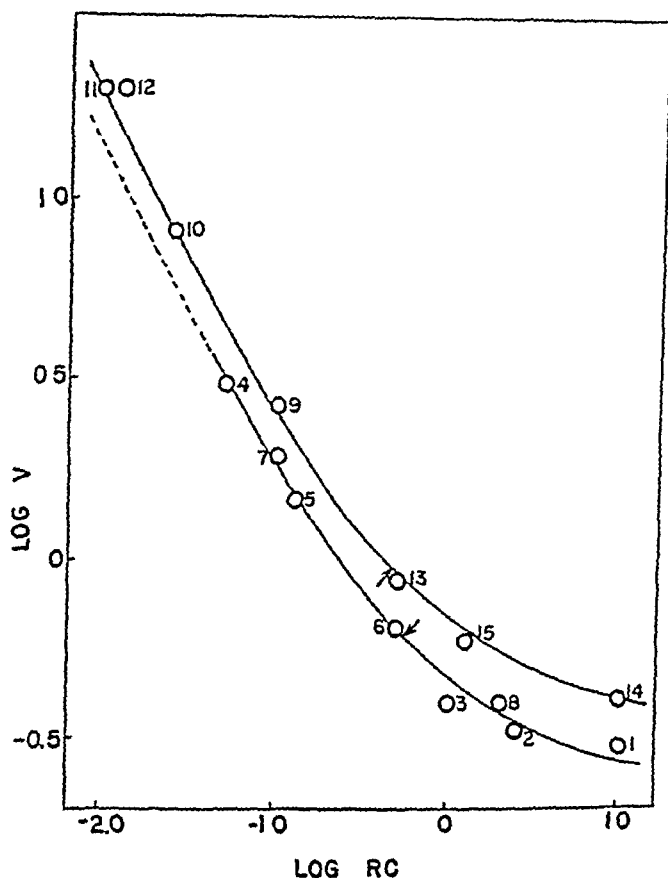


FIG 3 Strength-duration curve of isolated giant fiber during deterioration. Numbers on points indicate order in which they were obtained. Same curve moved to fit early and late data. Note shift of  $K$  value. Calomel electrodes, interelectrode distance 13 mm.

The characteristic times in intact trunks with electrode separations of 12-15 mm ranged from 0.40 to 0.46 and averaged 0.43 msec, while at 2-3.5 mm in the same preparations they averaged 0.33 msec (range 0.24-0.41). Similarly in four experiments on isolated giant fibers with interelectrode distances of 10-15 mm the characteristic times averaged 0.51 msec (range 0.46-0.58) while the same isolated

fibers with the same electrodes 3 mm apart showed characteristic times averaging 0.38 msec (range 0.16–0.42)

On the probability plots the curves with greater electrode separations have a lower slope and higher midpoint or modal value than

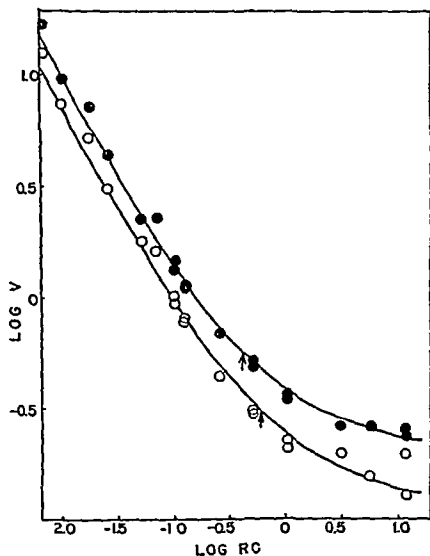


FIG. 4. Effect of electrode separation upon strength-duration curve of an isolated giant fiber. Lower curve (open circles) interelectrode distance 9.5 mm, upper curve (closed circles) electrode separation 1.5 mm. Curve of Hill's equation shifted to fit both sets of data.

those with electrodes close together. This higher value with greater electrode separations for the point where  $\frac{E_0}{E} = 50$  per cent was predicted by Crozier (1937) on the basis of making available a larger population of excitable elements.

*Effect of Electrode Size*

The relation between electrode size and excitation time was investigated in the experiments in which silver-silver chloride electrodes were used. Data on the giant fibers obtained with silver wire electrodes 0.6 mm in diameter and pore electrodes less than 0.1 mm in diameter gave similar results, the fibers tested being 0.2 to 0.7 mm

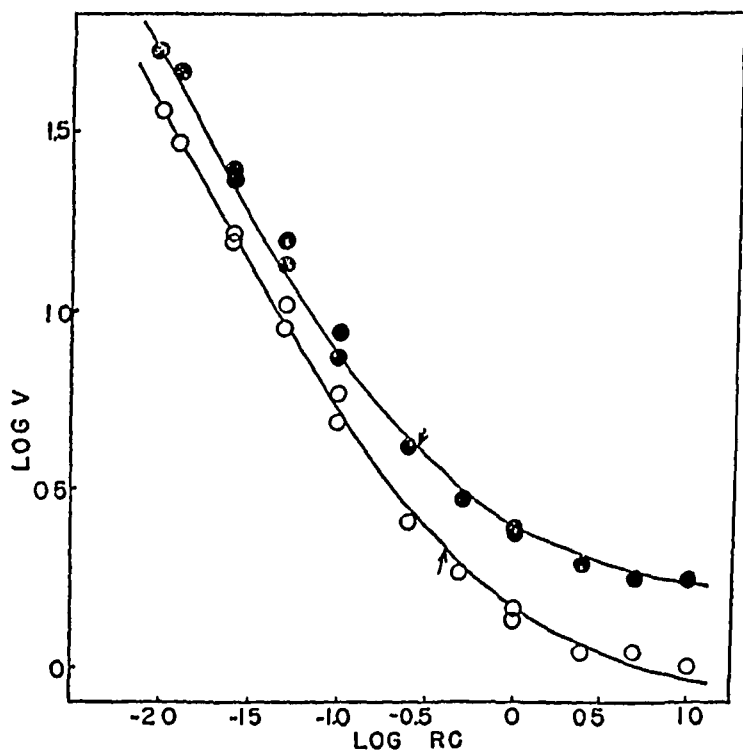


FIG. 5 Effect of electrode separation upon strength duration curve of a giant fiber intact in great stellar nerve. Lower curve (open circles) electrode separation 15 mm, upper curve (closed circles) electrode separation 3 mm. Note shift of Hill's  $K$  value as indicated by arrows.

in diameter. The characteristic times with wires are 0.13 to 0.70 and with pore electrodes 0.34 to 0.66 msec. This shows that the wire electrodes acted as small electrodes for these huge fibers. Only when larger chlorided silver plate electrodes 7 mm in diameter were used did longer excitation times appear. With the plates characteristic times ranged from 0.45 to 1.2 msec. The ranges of characteristic times with the large and small electrodes overlap considerably.

and the results are less dependable than in the experiments in which calomel electrodes were used

### *Effect of Fiber Diameter*

The fibers of the fin nerves are very much smaller in diameter than the giant fibers, and range from 1 to 50 micra in diameter They

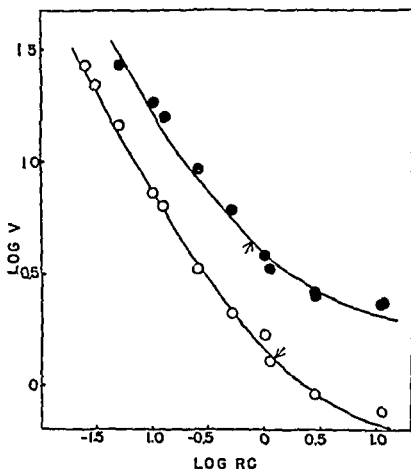


FIG 6 Strength duration curves of one fin nerve Calomel electrodes Lower curve (open circles)—interelectrode distance 12.5 mm Upper curve (closed circles) electrode separation 3 mm Solid lines—plots of Hill's equation  $K$  values indicated by arrows

yield consistently longer time constants than do the giant fibers Fig 6 shows strength duration curves for low threshold fibers of one fin nerve with calomel electrodes at 12.5 and 3.0 mm separation The characteristic times are 1.2 and 0.77 msec respectively Fig 7 is a curve obtained with chlorided silver wire electrodes 3 mm apart In a series of experiments with silver-silver chloride electrodes the characteristic times ranged between 0.92 and 2.4 msec These values

are two to five times longer than with the giant fibers. The variability of the data from the fin nerves is greater than with the giant fibers because the end-point of the contraction is not so sharp, but all of the excitation curves lie well to the right of those for the giant fibers. Rosenberg (1935) obtained similar values (1.4 msec) for the characteristic time in the fin nerve of *Sepia* which contains fibers up

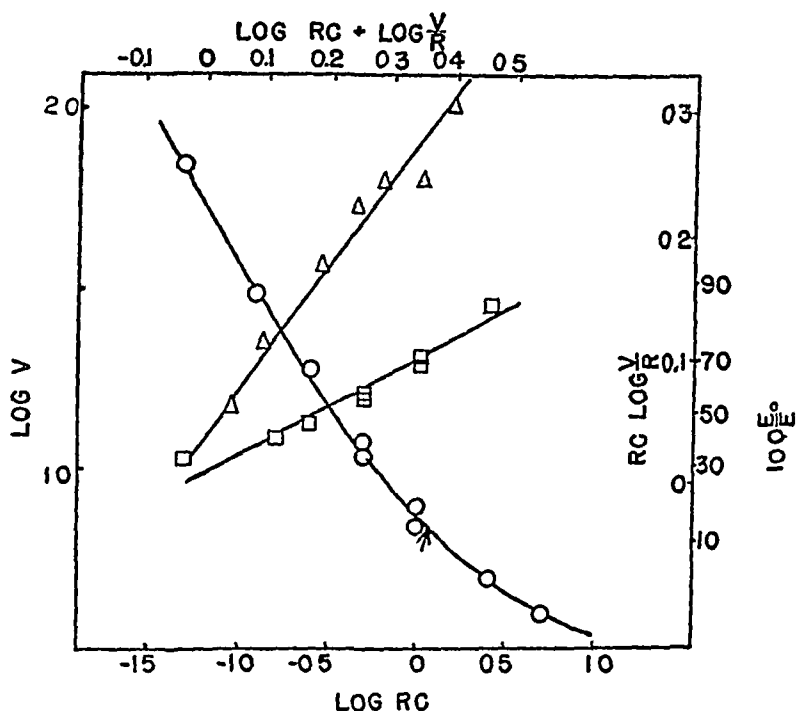


FIG 7 Strength-duration curve of low threshold fibers in fin nerve. Chlorided silver wire electrodes. Curve is plot of Hill's theoretical equation. Lower straight line (squares)—probability plot, ordinates at extreme right. Upper straight line (triangles)—plot of Blair's equation, abscissae at top, ordinates inside at right.

to 35  $\mu$  in diameter (Young, 1936 c), and Bugnard and Hill (1935), using the same nerve, obtained values of 2.5 msec when electrodes were separated by 1.5 mm and of 4 msec when they were separated by 20 mm.

#### "Subordination"

There is no indication of any "subordination" effect. In a series of fourteen experiments in which chlorided silver wire electrodes were

used, the chronaxies for the uncut giant fibers of the stellar nerves were between 0.05 and 0.37 and for the cut ones between 0.05 and 0.12 msec. Chronaxies for uncut fin nerves fell between 0.28 and 0.59 msec. and for cut ones between 0.43 and 0.56 msec. (except 1). However, the ganglia of the squid are highly sensitive to oxygen lack and it cannot be assumed that under the conditions of these experiments the cell bodies were in a normal condition. It is not clear what factors are responsible for the discrepancy between the results here obtained and those of Lapicque (1929) who found chronaxies of 2-3 msec. in the great stellar nerve of *Loligo*.

#### SUMMARY AND CONCLUSIONS

1 Strength duration data for the giant fiber of the great stellar nerve of the squid (*Loligo pealii*) can be approximately described by several mathematical formulations.

2 Excitation time constants for isolated giant fibers are essentially the same as constants of the giant fibers in the intact nerve.

3 The strength duration curves of the fibers in the intact nerve lie higher on the voltage axis than those of the isolated fibers. It is concluded that the principal effect of other fibers upon the excitation of one fiber in a nerve trunk is that of shunting the stimulating current.

4 Deterioration of the nerve shifts the curve upward and to the left, resulting in shorter time constants.

5 Decreasing interelectrode distance also shifts the curve upward and to the left.

6 Excitation time constants of the giant fibers are larger with plate electrodes than with wire or pore electrodes.

7 The strength duration curves of the smaller fin nerve fibers lie consistently to the right of, and the time constants are longer than those of the giant fibers.

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# ON RHODOPSIN IN SOLUTION

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## I

### INTRODUCTION

Three photolabile retinal pigments are now known rhodopsin, porphyropsin, the visual purple of fresh water fishes (Köttgen and Abelsdorff, 1896, Wald, 1937*b*), and iodopsin, a pigment of the cones (Wald, 1937*c*)

In the experiments to be described, solutions of rhodopsin have been examined from four species of marine fish—the sea robin, *Prionotus carolinus*, sea bass, *Centropristes striatus*, scup, *Stenotomus chrysops*, and killfish, *Fundulus heteroclitus*, two species of frog—*Rana pipiens* and *catesbeiana*, rabbits and rats

It has already been shown in all of these animals but *Fundulus* that rhodopsin participates in a retinal cycle which includes the carotenoids retinene and vitamin A (Wald, 1935–36, 1936–37, unpublished observations on mammals) The porphyropsin system is based upon other carotenoids, probably the same is also true of iodopsin The association with retinene and vitamin A is therefore a specific characteristic of the rhodopsin system

The spectrum of rhodopsin is equally characteristic A representative collection of spectra of rhodopsin preparations from various animals, and for comparison a typical porphyropsin spectrum, are shown in Fig 1 It is clear that the rhodopsin spectra form a distinct and homogeneous group with a single broad maximum at  $500 \pm 2 \mu$ <sup>1</sup>

\* This research has been aided in part by a grant from the Milton Fund of Harvard University A short account of the experiments has appeared in *Nature* (1937*a*)

<sup>1</sup> Bayliss, Lythgoe and Tansley (1936) have reported that preparations of visual purple from a number of marine fishes possess spectra intermediate between those of rhodopsin and porphyropsin The significance of these observations is

Rhodopsin therefore is clearly characterized chemically and spectroscopically and may be treated as a chemical individual without special reference to its source

Kuhne<sup>2</sup> described the bleaching of rhodopsin in solution as a wholly photo-chemical transformation in two stages (1) the formation of a yellow product with increased absorption in the violet, and (2) the slow fading of this product on prolonged irradiation to a final condition "colorless, like water" The second process appears to be a common photo-oxidation It is independent of the first reaction, probably possesses no significance for the retinal cycle, and is disregarded in the present paper

The denial of the first of these stages by Kottgen and Abelsdorff and by Trendelenburg (1904) led to an extended theoretical excursion If, as these authors believed, rhodopsin bleaches directly to colorless products, the final absorption of a bleached preparation may be assumed due to impurities present in the original solution The difference between this residual absorption and the spectrum of the unbleached preparation should constitute the absorption spectrum of pure rhodopsin Such "difference spectra" were found to agree in form with the sensitivity to monochromatic lights of rhodopsin solutions (Trendelenburg) and of rod vision (König, 1894, Trendelenburg, 1904, Hecht and Williams, 1922-23)

The assertion that rhodopsin bleaches to colorless products rested on wholly inadequate evidence Garten (1906) and more recently Hosoya and Bayerl (1933),<sup>3</sup> have entirely confirmed Kuhne's description As Garten indicated, Kottgen and Abelsdorff's own results contain abundant evidence of the formation of yellow products in bleaching Their conclusion to the contrary seems to have

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not clear to me Unfortunately the authors measured, not the direct absorption spectra, but differences in absorption between unbleached and bleached solutions This may at least in part account for their results It is noteworthy that one of their fishes showing apparently typical porphyropsin behavior is a gurnard, *Trigla hirundo*, our closely related American gurnard, *Prionotus*, possesses a typical rhodopsin system I have suggested the possibility that the English preparations contained mixtures of rhodopsin and porphyropsin (Wald, 1937 *b*), but this is excluded in the present experiments by the carotenoid extraction data, which show the presence of vitamin A and retinene alone (Wald, 1936-37)

<sup>2</sup> Kuhne, 1878, p. 49, 1879, p. 269

<sup>3</sup> It is noteworthy that these authors "neigen—der schon von Kottgen und Abelsdorff geauszerten Meinung zu, dass es sich bei dieser Ausbleichung nicht um die Entstehung eines neuen Farbstoffes handelt, sondern lediglich um das Verschwinden des roten Farbstoffes, ein Vorgang der nach unserer Meinung gut durch eine im Licht stattfindende physikalisch-chemische Veränderung, z B eine Dispersitäts-Änderung des Sehpurpurs erklärt werden kann"

been influenced by two attractive considerations (1) the possibility of deriving by simple spectrophotometric manipulation 'pure' absorption spectra from impure preparations and (2) the close correspondence between such difference spectra and human rod visibility. For a long period this comparison constituted the only direct evidence that rhodopsin is a *visual* pigment.

It is now clear that under all known conditions the bleaching of rhodopsin yields colored products (Lythgoe 1937, Wald 1937*a*). Difference spectra therefore though on occasion useful for descriptive purposes possess no physical meaning. The absorption spectrum of pure rhodopsin is still unknown.

Recently the belief that rhodopsin bleaching is exclusively photochemical has been questioned due to the discovery that following irradiation, dried gelatin films of rhodopsin (Weigert and Nakashima 1930) and aqueous solutions (Hosoya 1933) continue to fade in darkness.

The present research is concerned with the course of bleaching of rhodopsin in aqueous solution, the contribution to it of thermal ("dark") processes, its products, and the form and significance of the rhodopsin absorption spectrum.

## II

### *Methods*

Rhodopsin solutions were prepared by leeching retinas of dark adapted animals for various periods at room temperatures with a 2 per cent aqueous solution of crystalline digitonin (Tansley 1931). Special care was taken during the dissection of the tissues to remove all apparent traces of choroid and pigment epithelium. Two general methods of preparation were employed.

1. Retinas were dropped directly or after preliminary soaking in 5 to 10 per cent salt solutions into digitonin. The mixture was left in darkness for several hours with no other disturbance than occasional gentle rotation and was then centrifuged. These preparations were slightly opalescent and displayed reasonably constant properties, the most specific of which was an extinction at 400  $m\mu$  about 0.5 to 0.6 of that at the maximum.

2. Directly following dissection retinas were dropped into 4 per cent aluminum potassium sulfate (alum) solution and left to harden for 3 hours or longer. The mixture was centrifuged, the alum solution poured off, and the tissues washed twice in distilled water, then twice more in buffer mixtures to bring to the desired pH. Finally they were ground with a glass rod in digitonin solution, let stand for several hours and centrifuged. These preparations appeared to be perfectly clear and were distinguished by extinctions at 400  $m\mu$  only 0.32 to 0.35 of those at the maximum.

Spectra were measured at the Color Measurements Laboratory, A. C. Hardy, at the Massachusetts Institute of Technology, with

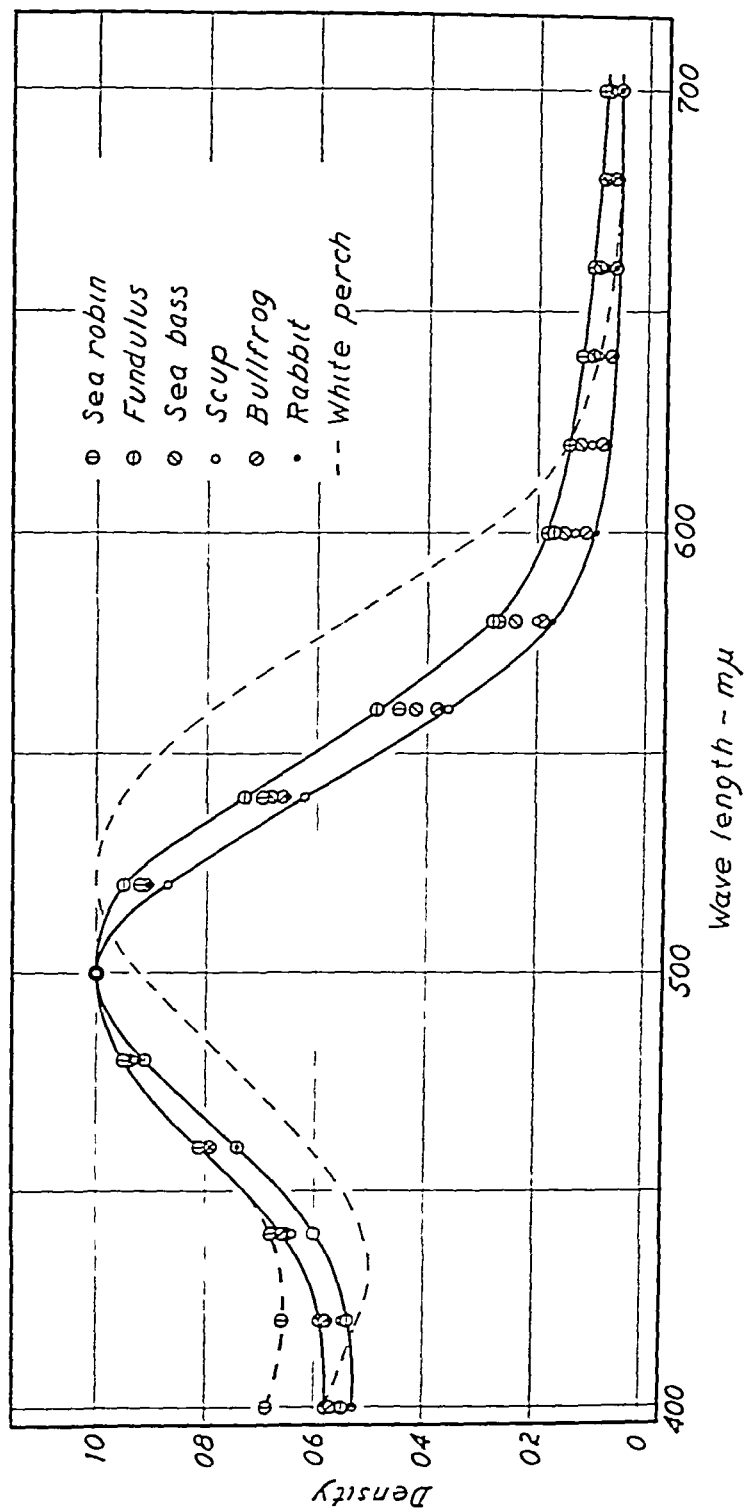


FIG. 1. Spectra of rhodopsin preparations from the rabbit, bullfrog, and four marine fishes, and of a typical porphyropsin preparation from the fresh water white perch (*Morone americana*). All but the bullfrog preparation were obtained from retinas pretreated with dim. The absorption is plotted as density or extinction,  $\log \frac{I_0}{I}$ , in which  $I_0$  is the incident and  $I$  the transmitted intensity.

photoelectric spectrophotometer (Hardy 1935) The spectra shown in Figs 2 3 and 5-8 were drawn by the instrument itself This machine in addition to complete objectivity in performance possesses two special advantages for the present research (1) it is extraordinarily rapid The spectrum from 400 to 700  $m\mu$  is recorded within about 2 minutes (2) It is economical of light Since the spectrum is dispersed at the source only the narrow wave length band the absorption of which is being measured passes through the test solution This causes no distinguishable bleaching of rhodopsin during the measurement of an entire absorption spectrum indeed the spectrum may be retraced completely without perceptible change in absorption With respect to the preparation therefore, the period of measurement is equivalent to a period in darkness

The general procedure in bleaching experiments was to measure the spectrum of the rhodopsin preparation then irradiate it in position in the spectrophotometer and re measure its spectra thereafter periodically without otherwise disturbing it

All of the spectra recorded in the following pages show the differences in absorption between test solutions and equal depths of pure solvent The absorption is expressed either as a percentage of the incident radiation or as extinction or optical density  $\log I/I$  in which  $I$  is the incident and  $I$  the transmitted intensity The latter quantity is directly proportional to the concentration and to the depth of absorbent

### III

#### *The Bleaching of Rhodopsin*

Rhodopsin bleaches in a succession of light and "dark"—photic and thermal—reactions<sup>4</sup> The velocity of the light component depends principally on the intensity of irradiation, that of the dark component on the temperature In dim light the light reaction may be so slow as to limit the speed of the entire transformation In this case which characterizes almost all earlier studies of bleaching, the contribution of dark processes is obscured and usually overlooked In the present experiments lights were used sufficiently bright to complete the photoprocess within a few seconds Following such exposure the isolated dark reactions may be examined through a long succeeding interval

The course of bleaching in neutral solutions of bullfrog rhodopsin is shown in Figs 2 and 3 It possesses the following components

*Photoprocess*—The first recorded product of irradiation is an

<sup>4</sup>Throughout this paper reactions are characterized as light or dark' synonymously with the more accurate terms photic and thermal Dark reactions of course proceed equally well in light or darkness

orange-colored pigment, the spectrum of which is shown in curves *b*. It possesses a broad absorption hump at about  $480\text{ m}\mu$ , and higher absorption than rhodopsin itself below  $430$  to  $440\text{ m}\mu$ . The change in extinction due to the photoprocess, revealed by subtracting curve *b* from *a*, may be plotted as a "difference spectrum." This

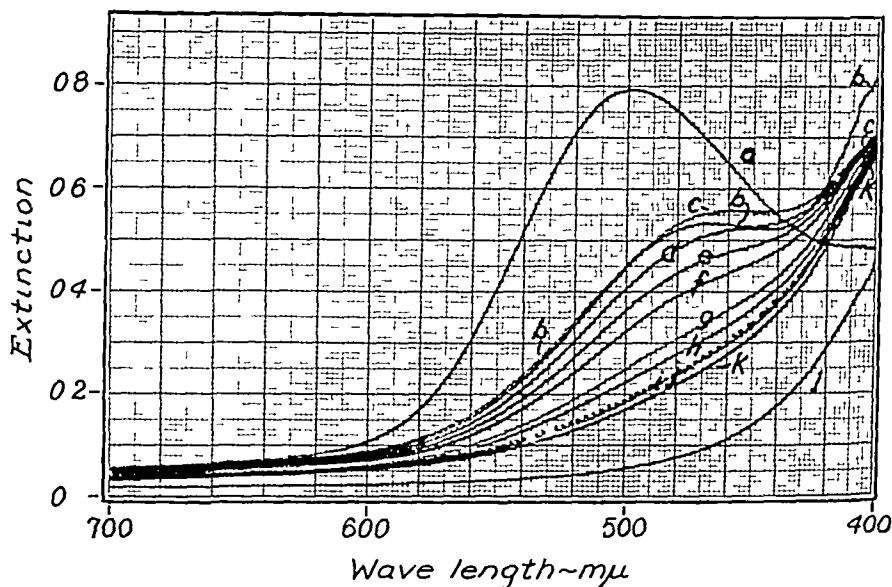


FIG 2 Bleaching of neutral rhodopsin. Extract of 6 bullfrog retinas in 3.8 cc of 1 per cent digitonin solution buffered at pH 6.9. Spectra of a 1 cm layer recorded at  $25.3^{\circ}\text{C}$ , 6 hours after the beginning of extraction. The unbleached solution (*a*) was exposed for 25 seconds to 700 foot-candles and spectra recorded periodically thereafter in the dark: *b* at 1.5 minutes, *c* at 4.5, *d* at 7.5, *e* at 10.5, *f* at 15.0, *g* at 32.3, and *h* at 59.5 minutes from the beginning of irradiation. At 61 minutes the solution was re-irradiated for 40 seconds, its spectrum fell to *i*. After a further 22 minutes in darkness it had risen to *j* (broken line), and on a third irradiation for 40 seconds dropped to *k*. The spectrum of the benzene-soluble portion of the final bleached residue in 3.8 cc of a mixture of 1 part methanol and 2.8 parts digitonin solution, buffered at pH 6.9, is shown in curve *l*.

has been done with the data of Fig. 3B, the result is shown in Fig. 4 (open circles). The fall in extinction due to light is maximal at about  $510\text{ m}\mu$  and is accompanied by a rise at  $400\text{ m}\mu$  which may be almost equally great.

Hosoya (1933, Hosoya and Bayerl 1933, Hosoya and Saito, 1935)

has reported that on irradiation of rhodopsin the absorption rises also in the red, above 600-620  $m\mu$ . This phenomenon has never appeared in the present experiments.

*Dark Process I*—This is shown in Fig. 2 curves *b* to *c*. The ex

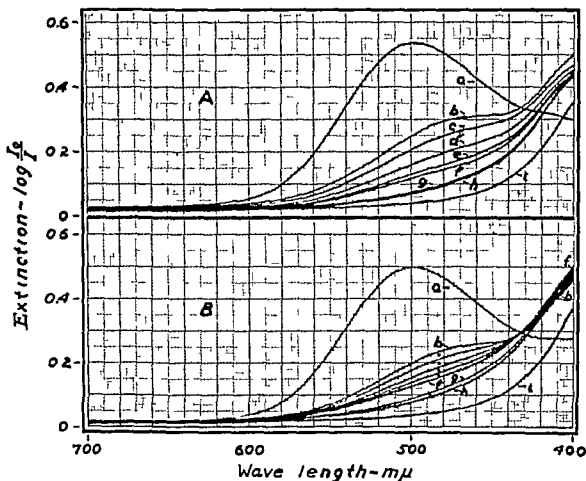


FIG. 3 Bleaching of neutral rhodopsin in fresh and aged solutions. Extract of 7 bullfrog retinas in 7.6 cc. of 1 per cent digitonin solution buffered at pH 6.9. Half of this preparation was measured 4 hours after extraction was begun (A); the remainder one week later (B). During the storage period of B a small amount of colorless material came out of solution and was centrifuged off. Spectra of a 1 cm. layer of the unbleached solutions are shown in curves *a*. After  $\frac{1}{2}$  minute exposure to 700 foot candles spectra were measured periodically in darkness: curves *b* at 1.2 minutes; *c* at 3.2 and 3.7; *d* at 7.2 and 8; *e* at 13.8 and 15.7; and *f* at 32.2 and 32.8 minutes (when two times are stated the first refers to A). At 35 minutes the preparations were reirradiated as before for 40 seconds and their spectra recorded (*g* 37 minutes). At 38.5 minutes they were exposed for a third time and yielded curves *h* (40 minutes). Temperatures: A 29.3°C; B 26.0°C. Curves *i* show the spectra of the benzene soluble portions of the bleached residues, each in 3.8 cc. of a mixture of 1 part methanol and 2 parts digitonin solution buffered at pH 6.9.

tion rises rapidly in darkness between about 420 and 500  $m\mu$  maximally at about 440  $m\mu$  and falls to either side of this region. This component is most prominent in the bleaching of acidic rhodopsin. It has not been detected at all in alkaline solutions, and often is missing from neutral preparations as in Fig. 3. When present it appears equally prominently in all samples of a preparation and apparently is unaffected by aging of the solution. I believe this to be a normal component of the bleaching of neutral rhodopsin and that it is missed in some preparations only because, for unknown reasons it passes too swiftly to be recorded.

*Dark Process II*—This is shown isolated in Fig. 3B, curves *b* to *f*. The extinction falls slowly between about 435 and 600  $m\mu$ , maximally at about 480  $m\mu$  and rises simultaneously below about 435  $m\mu$ . The solution changes in color from orange to yellow, as the initial photoproduct maximum at 480  $m\mu$  is entirely obliterated. The difference spectrum of this change, obtained by subtracting curve *f* from *b* in Fig. 3B, is shown in Fig. 4 (closed circles). It resembles closely that due to the photoprocess except for the shift of maximum from about 510 to about 480  $m\mu$ . This dark process is an invariable component of the bleaching of neutral rhodopsin. In Fig. 3B it accounts for 19 per cent of the total fall in extinction from curves *a* to *f* at 500  $m\mu$  and 26 per cent of that at 480  $m\mu$ .

*Dark Process III*—Hosoya (1933) discovered a slow, general fall in the entire spectrum of bleached products of rhodopsin following prolonged low intensity irradiation. I find that this phenomenon appears clearly only in fresh neutral preparations. It is prominent in Figs. 2 and 3A recorded within several hours following extraction, but has vanished completely after a week of storage (Fig. 3B). This component is not prominent even in fresh solutions from retinas pre-treated with alum or at pH's below 5.

A difference spectrum showing the changes in extinction due to the combination of this process with dark component II is obtained by subtracting curve *f* from *b* in Fig. 3A. This is shown in Fig. 4 (half-filled circles). The fall in extinction is maximal at about 480  $m\mu$ , and no further rise occurs at 400  $m\mu$ .

*Contribution of Dark Processes to Bleaching*—Of the total fall in extinction from curves *a* to *f* in Fig. 3A 31 per cent at 500  $m\mu$  and



42 per cent at  $480\text{ m}\mu$  are due to dark processes. The corresponding values from Fig 2 are 40 and 52 per cent. In a rhodopsin preparation which had been exposed to the 0.02-0.04 second flash of a magnesium foil lamp (Photoflash No. 20) the fall in extinction at  $500\text{ m}\mu$  during a subsequent 1.2 to 98 minutes in darkness was 48 per cent of

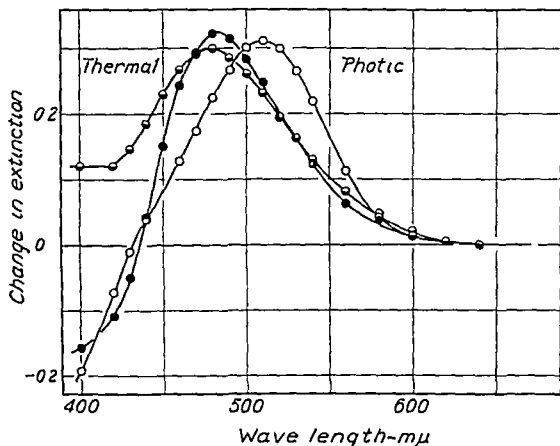


FIG 4. Difference spectra of rhodopsin from the data of Fig 3. Open circles show changes in extinction due to the photoprocess (Fig 3 B a-b). Half closed circles show those due to thermal processes in fresh solutions (Fig 3 A b-f), closed circles those due to the thermal process in aged solutions (Fig 3 B b-f). To make these curves conveniently comparable the ordinates of the second function have been multiplied throughout by 2, those of the third by 4. Bleaching due to light is maximal at about  $510\text{ m}\mu$ ; that due to thermal reactions at about  $480\text{ m}\mu$ .

the total (22.4°C). The contribution of dark processes to bleaching actually rises above any of these estimates, since it includes changes which occur before the first and following the final measurement, and some correction for whatever regeneration of rhodopsin may have occurred in the dark period. It may be concluded that in the bleaching

This is clearly analogous to thermal component I of bleaching in neutral solution. In acid solution it appears invariably and very prominently, and its velocity is greatly retarded.

This process is followed by a slow, regular fall in absorption, maximal at  $460\text{ m}\mu$ , with simultaneous rise below about  $416\text{ m}\mu$ . This is the acidic analogue of thermal component II in neutral solution, at

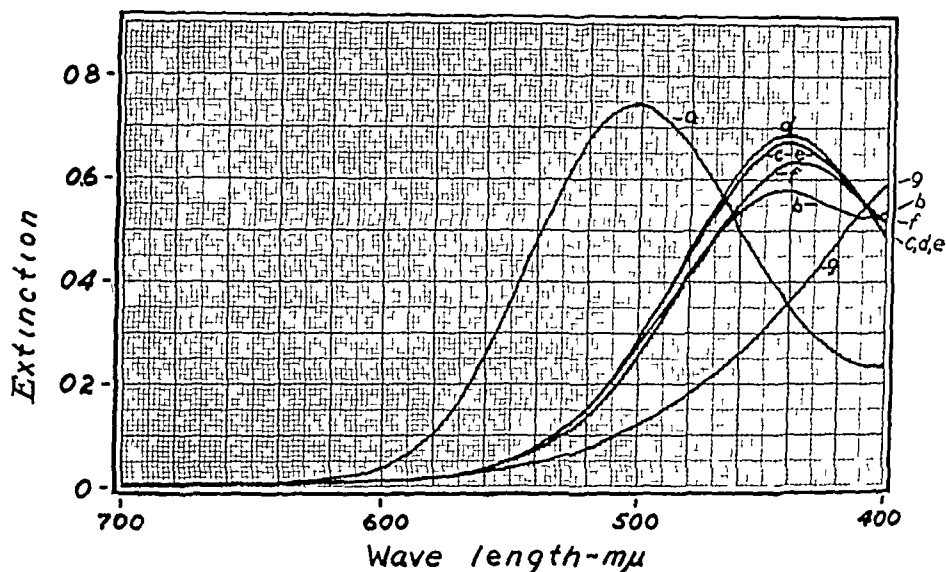


FIG 6 Acidic rhodopsin, alum preparation. 6 bullfrog retinas soaked in 5 per cent alum for 3 days at  $5^{\circ}\text{C}$ . Washed with water, soaked in 10 per cent saline for 3 hours, then ground in 3.8 cc of digitonin solution. The pH of the extract, measured before and after bleaching, remained constant at 3.9. The preparation had been at this pH a total of 6 days. Spectra of a 10 mm layer are shown. The unbleached solution (a) was exposed to 700 foot-candles for 30 seconds, and spectra recorded in darkness, b at 1.3 minutes, c at 3.8, d at 8.2, e at 15 (retraces c), f at 34.3 minutes from the beginning of irradiation ( $25.9^{\circ}\text{C}$ ). Final solution left in darkness for 28 hours longer (g).

pH 5 it is shifted about  $20\text{ m}\mu$  toward shorter wave lengths, and has ceased to be the most prominent dark factor in bleaching.

At higher acidities the peculiarities of Fig 5 are accentuated. The bleaching of rhodopsin at pH 3.9 is shown in Fig 6. The initial photoproduct (b) possesses a distinct maximum at about  $443\text{ m}\mu$ . During the first 8 to 10 minutes in darkness this rises rapidly, finally assuming a quite symmetrical form (c-f). Simultaneously the photo-



complete and not simply retarded in some intermediate condition, this solution was rapidly neutralized with a prepared quantity of monopotassium phosphate, and its spectrum remeasured within 4 minutes following neutralization (*d*), and again 9 minutes later (*e*), all in darkness. A very small fall in absorption at about  $480\text{ m}\mu$  and rise below about  $450\text{ m}\mu$  occurred from curves *d* to *e*, but these changes are almost negligible compared with those occurring in a comparable period in neutral solution. One may conclude that at pH 11 thermal components of bleaching are almost complete within 1 minute following short exposure to light.

In several bleaching experiments with rhodopsin at pH about 9, the thermal fall in extinction in the region of  $480\text{ m}\mu$  with simultaneous rise at  $400\text{ m}\mu$  characteristic of neutral solutions (dark process II) was found to be still prominent, though greatly increased in speed. We may assume that at pH 11 the normal sequence of changes during bleaching is unaltered, but that due to their very high velocity, intermediate stages are not recorded.

### *Recapitulation*

Four components have been distinguished in the bleaching of rhodopsin solutions

1 Photic reaction. In neutral solution the absorption falls maximally at about  $510\text{ m}\mu$ , simultaneously rising below a node at about  $430\text{--}440\text{ m}\mu$ . In alkaline solutions the node is displaced toward  $410\text{ m}\mu$ , in acidic solutions toward  $470\text{ m}\mu$ .

2 Thermal component I. In neutral solution the absorption rises maximally at about  $440\text{ m}\mu$ , simultaneously decreasing to either side of nodes at about  $420$  and  $495\text{ m}\mu$ . Above pH 7 this component has not yet been recorded. With increase in acidity it grows increasingly prominent, its velocity falls sharply, and the nodes are displaced toward shorter wave lengths.

3 Thermal component II. In neutral solutions the absorption falls maximally at about  $480\text{ m}\mu$ , and rises below a node at about  $435\text{ m}\mu$ . This process is recorded at pH's 9 and below. Its velocity decreases markedly with increase in acidity, simultaneously the region of maximal bleaching and the node are displaced toward shorter wave lengths.

4 Thermal component III The absorption of the bleached products decreases throughout the spectrum This change appears prominently only in fresh neutral preparations

Within this succession of photic and thermal reactions, the latter account for about half the total fall in extinction at 500  $m\mu$  Bleaching as a whole may be regarded as a slow explosion, touched off by an appropriate quantity of light in as short a period as desired, and proceeding to completion on internal energy reserves during a long subsequent interval

Though the spectrum of rhodopsin itself is unaffected by pH changes the spectra of all stages of bleaching are highly pH labile Rhodopsin apparently does not possess an acidic or basic grouping in close association with its chromophore but such a grouping is exposed as a first result of irradiation (*cf* Lythgoe, 1937) The effects of pH change on the absorption spectra of bleached products are in some cases large and peculiar probably because they involve complicated mixtures of pigments

#### IV

##### *Final Products of Bleaching*

The final yellow product of bleaching of rhodopsin in solution owes its color to a benzine soluble pigment most of which is attached loosely to protein It is extracted by the following procedure

To the yellow bleached residue an equal volume of acetone or alcohol is added to precipitate proteins On centrifuging most of the yellow color descends with the precipitate The precipitate is shaken with benzine<sup>5</sup> containing about 1 per cent ethanol, the solution separately with benzine All color enters the benzine extracts

The benzine extract is faint greenish yellow in color Partitioned between benzine and 90 per cent methanol, either before or after saponification about three fourths the pigment seeks the benzine layer (epiphasic) In chloroform its absorption rises regularly throughout the visible spectrum and possesses a small inflection at the edge of the ultraviolet which develops into a broad maximum at about 385  $m\mu$  Tested in chloroform solution with saturated antimony

<sup>5</sup> Petrol ether boiling range 50 to 60 C

trichloride, it yields a deep blue color due to a sharp band at  $664\text{ m}\mu$ . These properties identify it as retinene (Wald 1935-36)

Retinene has so far been examined only in impure condition. It is impossible, therefore, to be certain that any two properties of its solutions are due to a single substance. I have defined retinene specifically by the antimony trichloride band at  $664\text{ m}\mu$ , one may assume tentatively that other properties which invariably and proportionately accompany this one are due to retinene. The yellow color is such a property. In a series of retinene preparations obtained at various times and by various procedures, the densities of the yellow color and of the antimony trichloride band have been measured with a Pulfrich photometer (Zeiss). These quantities are directly proportional to each other. If a solution in chloroform possesses a density of 1.0 measured with the S43 filter in a layer 1 cm. in depth, then 0.3 cc. of it mixed with 3 cc. of antimony chloride reagent possesses an immediate extinction with the S66.6 filter of about 0.4 in a 1 cm. layer. This is true of extracts of retinas and of bleached rhodopsin solutions before and after saponification, and of all fractions of the extracts in partition between benzene and 90 per cent methanol.

It is shown below that a third property, change in color with pH, accompanies these solutions. I see no better alternative at present than to ascribe this also to retinene. It is an unusual property in a carotenoid, and therefore particularly interesting that it is exhibited also, though less markedly, by the tetra-keto  $\beta$  carotene, astacene. I find astacene in acidic methanol solution to be deep orange red in color, and to shift in hue markedly toward yellow in alkaline solution. Spectrophotometric measurements show this change to be due to a displacement of the entire spectrum 4 to  $8\text{ m}\mu$  toward shorter wave lengths. The long wave length limb of the astacene absorption band—comparable with the portion of the retinene spectrum measured in the present experiments—changes with pH as does retinene itself.

In the intact retina, retinene is converted quantitatively to vitamin A, characterized by an antimony trichloride band at about  $615\text{ m}\mu$  (Wald, 1935-36). This process occurs only slightly if at all in rhodopsin solutions. The antimony trichloride test with extracts of bleached solutions does possess a small hump at about  $615\text{ m}\mu$  superimposed on the high  $664\text{ m}\mu$  retinene band. This, however, has appeared heretofore in all retinene tests, and even if due wholly or in part to vitamin A is still no higher than occurs in extracts of dark adapted retinas (Wald, 1935-36). There is no present evidence that vitamin A is formed in the bleaching of rhodopsin solutions.

The benzene-soluble pigment from bleached rhodopsin exhibits the

color properties and pH changes of the entire residue. Spectra of a methanol solution of this pigment are shown in Fig 8, (a) neutral, (b) saturated with carbon dioxide at 25°C (c) in 0.25 N acetic acid, (d) in 0.56 N acetic acid, (e) in 0.6 N ammonia and (f) in 1.3 N ammonia. Clearly the pH lability of the final product of rhodopsin bleaching is due principally to this lipoidal pigment.

To compare accurately the spectrum of the benzene soluble pigment with that of the whole bleached residue, both solvents and pH should be identical. This condition is approximated by diluting

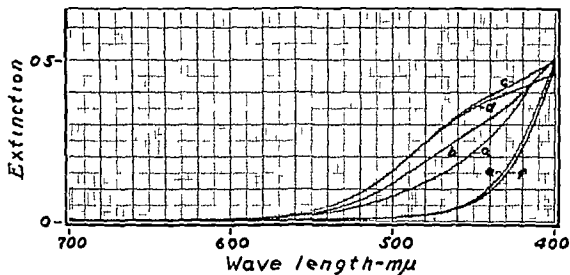


FIG 8 Benzene soluble pigment from the final residue of bleaching. Spectra of methanol solutions (a) neutral (b) saturated with carbon dioxide (c) in 0.25 N acetic acid (d) in 0.56 N acetic acid (e) in 0.6 N ammonia and (f) in 1.3 N ammonia. To bring to equivalent concentrations of pigment multiply all ordinates of (c) by 1.02 (d) by 1.04 (e) by 1.04 and (f) by 1.1.

methanol solutions of the lipoidal pigment with buffered digitonin solution. The latter alone can dissolve small concentrations of retinene forming solutions which differ very little spectroscopically from methanol solutions.

Curves *l* in Fig 2 and *i* in Fig 3 show spectra of the benzene soluble fractions of final bleached residues, brought to the original volumes in mixtures of methanol and aqueous digitonin at pH 6.9. Such solutions resemble very closely the whole residues but display considerably lower absorptions. The whole residues contain, in addition to benzene soluble pigment and salts, various proteins, including

the protein component of rhodopsin itself, which absorb and scatter light principally at low wave lengths. The difference in extinction between the whole residue and its benzene-soluble fraction is a measure of the apparent absorption due to these non-lipoidal constituents. This matter is discussed in detail below.

*State of Retinene in the Bleached Residue* —I have assembled elsewhere the evidence that rhodopsin is a conjugated protein, containing retinene or its carotenoid precursor as prosthetic group (Wald, 1935-36). At the close of bleaching the color and behavior of the residue closely resemble those of free retinene. The following experiments were undertaken to examine the association of retinene with protein in rhodopsin and in bleached solutions.

Free retinene dissolves very readily in benzene. Irradiated retinas yield their retinene quantitatively on simply shaking with this solvent. One cannot proceed so simply with rhodopsin solutions, to avoid the formation of viscous emulsions a special procedure is needed. Acetone quickly destroys rhodopsin at room temperatures liberating its retinene content in the process. I have found, however, that rhodopsin may be precipitated from solution quantitatively in the cold by adding an equal volume of acetone, with little immediate injury to the molecule. Precipitate and supernatant solution may be extracted separately in the cold with benzene or with benzene-acetone mixtures, still without decolorizing the rhodopsin. By this means the state of retinene may be examined in solutions of rhodopsin and of its bleached products.

When rhodopsin is precipitated in the cold with acetone practically all color descends with the precipitate. If precipitate and solution are extracted in the cold, the combined extract contains a very small amount of yellow pigment. This has never been obtained in quantities sufficient for identification. Its spectrum displays simple end-absorption. It may be retinene or some lipoidal impurity of the original solution. Its density at 400  $m\mu$  is only about 5 per cent of that of the total benzene-soluble fraction of the bleached residue.

The addition of an equal volume of acetone to a bleached solution yields a precipitate containing protein and most of the yellow color. The retinene which descends with this precipitate must be attached



to protein in the final bleached product. Free retinene would not be precipitated by addition of acetone, and the possibility that the retinene is adsorbed and carried down during the formation of the protein precipitate must be rejected, since carotenoids are not ordinarily adsorbed appreciably in the presence of high concentrations of acetone.

The extraction of retinene from this precipitate with benzene is subject to two conditions: (a) the benzene must contain a small quantity (1 to 2 per cent) of acetone or alcohol, preferably the latter, and (b) the extraction is greatly retarded at low temperatures. This behavior suggests two possibilities: (1) Retinene may remain in part adsorbed on the protein residue of the rhodopsin molecule after bleaching has broken its chemical bond. Small quantities of the typical carotenoid eluents, alcohol and acetone, liberate retinene by simple displacement from the adsorbent. (2) Retinene may remain in part chemically bound to protein in the final bleached product. Alcohol and acetone disrupt this complex just as they destroy rhodopsin itself in the warm, by attacking the protein.

By adding equal volumes of acetone to rhodopsin solutions in various stages of bleaching, and determining separately the retinene contents of the precipitate and of the supernatant solution, one may estimate the extent to which retinene is liberated during bleaching. Immediately following irradiation about five sixths of the retinene is found to descend with the protein precipitate, and after all thermal components of bleaching are complete this fraction has fallen only to about two thirds.

The final product of bleaching of rhodopsin in solution is therefore a mixture of retinene and protein, about two thirds still loosely attached to each other in some manner that scarcely alters the retinene spectrum. It is this mixture to which Kuhne at times applied the term "visual yellow," and which Lythgoe (1937) has suggested be called "indicator yellow." Both terms are ambiguous. The former has been applied at various times to probably all orange or yellow products of bleaching in both retinas and solutions, the latter fails to distinguish the final mixture from intermediate yellow or orange products of bleaching, all of which appear to be pH indicators.

## V

*The Spectrum of Rhodopsin*

Rhodopsin solutions are principally contaminated with yellow and colloidal impurities, both of which, the former by absorption, the latter by differential scattering, raise the apparent extinction principally in the blue and violet. The ratio of the extinction at  $400\text{ m}\mu$  to that at the maximum,  $500\text{ m}\mu$  ("400/500 ratio"), therefore offers a convenient criterion of the purity of a preparation. The lower this ratio, the purer is the solution.

My preparations form two groups in this respect. Solutions of rhodopsin from bull frog retinas extracted directly or after preliminary soaking in brine exhibited 400/500 ratios of 0.52–0.59. Preparations from other animals, derived from retinas pretreated with alum, also fall into this group. A representative series of such spectra has been shown in Fig. 1. The relative constancy of these spectra in spite of considerable variation in the details of preparation suggested for a time the possibility that the solutions were approximately pure.

However, almost simultaneously a number of investigators including myself prepared rhodopsin solutions possessing 400/500 ratios of 0.30 to 0.35. These were derived from retinas pretreated with acid (Lythgoe, 1937), or with acidic alum solutions (Chase and Haig, 1937–38, present experiments), or by purifying initial extracts by fractional precipitation (Krause and Sidwell, 1938). The efficacy of alum treatment appears to be due to the fact that proteins other than rhodopsin are rendered insoluble. It is possible that rhodopsin itself is significantly changed by this treatment, but no specific difference in behavior has yet been detected between these and non-alum-treated preparations. For the present it may be assumed, therefore, that alum treatment does not significantly affect those properties of rhodopsin with which we are concerned. The spectrum of my best preparation from alum-treated retinas is shown in curve *a* of Fig. 6. Its 400/500 ratio is 0.32.

These rhodopsin solutions still contain unknown quantities of impurities, some of which may contribute to their absorption in the visible spectrum. It is possible, therefore, that the spectrum of pure rhodopsin lies considerably below that of any preparation yet meas-

ured There is a strong disposition to believe that the pure rhodopsin spectrum is identical in form with the visibility function of the rods, in this case its absorption at  $400\text{ m}\mu$  should be only about 5 per cent of that at the maximum (*cf* Lythgoe, 1937)

Hosoya and Bayerl (1933) have attempted to estimate empirically the absorption due to impurities in rhodopsin solutions From the spectrum of a rhodopsin preparation they subtracted the spectrum of an extract of light adapted retinas prepared in the same way The resulting curve was unsymmetrical, maximal at about  $500\text{ m}\mu$ , and possessed a 440/500 ratio of 0.47 If this computation is reliable, the spectrum shown in curve *a* of Fig. 6 approximates that of pure rhodopsin, for its 440/500 ratio is almost precisely 0.47

Hosoya and Bayerl's procedure, however, is open to serious question The spectra of all their rhodopsin preparations indicate the presence of large and variable quantities of impurities Presumably, control extracts of light adapted retinas varied similarly Large inaccuracies might consequently be expected to accompany estimation of the impurities in one preparation by those found in another Only a single experiment of this sort is mentioned, and its outcome might possibly have been largely fortuitous

With the aid of data presented in the foregoing pages the spectrum of pure rhodopsin may be established within narrow limits Its upper limit obviously is the directly measured total absorption of a rhodopsin preparation Its lower limit is fixed by the following procedure

It is assumed that during the bleaching of rhodopsin in solution no other colored component changes its spectral characteristics The final bleached residue contains newly formed retinene and protein, in addition to impurities present in the original solution To a first approximation, retinene is the only benzene soluble pigment in this mixture This is extracted and its spectrum measured in the original volume of solvent The difference between its absorption and that of the entire bleached residue is due to the protein product of bleaching and to impurities present in the original solution This difference, therefore, sets an upper limit to the absorption in the original solution which may be ascribed to impurities or to the absorption or scattering of light by the protein fraction of rhodopsin itself If this difference is subtracted from the spectrum of the rhodopsin preparation, the

resulting curve is the lower limit for the absorption of pure rhodopsin, or, expressed more precisely, the lower limit of absorption of the rhodopsin chromophore

An example may clarify this procedure. Curve *a* of Fig 3 B possesses a 400/500 ratio of 0.56. The difference in absorption between its final bleached product (curve *b*) and its benzene-soluble fraction in clear solution (curve *c*) constitutes the upper limit of absorption in the bleached residue due to impurities and scattering of light by colloidal particles. If this difference is subtracted from spectrum *a*, the approximate lower limit of pure rhodopsin absorption is obtained. Its 400/500 ratio is 0.335. In five other preparations a similar procedure has yielded lower limit curves with 400/500 ratios of 0.30 to 0.34, with an average value of 0.32. The 440/500 ratios of these functions are close to 0.47, agreeing very well therefore with Hosoya and Bayerl's corrected spectrum and with the uncorrected spectra of preparations from alum-treated retinas.

This approximate calculation, however, requires some further adjustment. Ideally the benzene-soluble fraction should contain only the retinene produced by bleaching in free solution in buffered, colorless, 1 per cent digitonin. Actually it includes all benzene-soluble substances in the final bleached residue, in a buffered solution of digitonin in methanol-water mixtures. The discrepancies between these situations are counterbalanced as follows:

1. The digitonin used to prepare the solutions of Fig 3 B itself absorbed light very slightly at low wave lengths. Its density in a 1.3 per cent solution—the concentration used in curve *c*—was measured and subtracted from curve *c*.

2. The benzene-soluble fraction in true solution in alcohol or chloroform does not absorb light appreciably at 700 m $\mu$ . In aqueous mixtures it does register a small absorption at this wave length, either real or due to scattering of light by the solution. I have compensated for the possibility that this absorption is an artifact by arbitrarily bringing the extinction at 700 m $\mu$  to zero, in the case of Fig 3 B subtracting 0.003 from all values of curve *c*.

When these two corrections have been made the 400/500 ratio of the lower limit of rhodopsin absorption in Fig 3 B has fallen to 0.27.

3. There is some doubt that the benzene-soluble residue is in true

solution in aqueous methanol digitonin mixtures. If this is really a colloidal emulsion or suspension, some of its apparent absorption might be due to scattering of light. Solution *z* in Fig 3B appeared clear to the eye, but on more rigid examination it might have revealed some optical heterogeneity.

These first three difficulties do not arise at all in two experiments in which the absorption of the benzene soluble pigment was measured in absolute methanol. Undoubtedly the solutions were "true," the methanol possessed no intrinsic absorption, and the extinctions of the solutions at 700  $m\mu$  were zero. The lower limits of rhodopsin absorption computed from these experiments possessed 400/500 ratios of 0.30. This is in good agreement with the above, probably somewhat over corrected result.

4. The final correction is most important. Benzene soluble colored substances may have been present in the original unbleached solution, and these must be estimated and reckoned among the impurities. For this purpose several rhodopsin solutions were precipitated with cold acetone and the precipitate and supernatant solution were extracted with benzene acetone mixtures in the cold, as described in the preceding section. The absorptions of such extracts in two experiments were found to be negligible at 500  $m\mu$ , and at 400  $m\mu$  were 4.4 and 5.6 per cent of the extinctions at 500  $m\mu$  of the original rhodopsin. One may therefore correct for this factor approximately by subtracting from the extinction at 400  $m\mu$  of, for example, curve *z* of Fig 3B, 5 per cent of the extinction at 500  $m\mu$  of curve *a*.

When this final correction has been applied to curve *z*, the difference between it and curve *h* represents the thoroughly corrected upper limit which may be assigned to absorption and scattering by impurities and by the protein component of rhodopsin. This absorption, subtracted from curve *a*, yields a lower limit spectrum for the pure rhodopsin chromophore possessing a 400/500 ratio of 0.20. Similar corrections applied to the two experiments in which the benzene soluble pigment from bleached residues was measured in methanol solution reduce their lower limit 400/500 ratios to 0.23 and 0.24.

Fig 9 shows the spectrum of the whole rhodopsin preparation, taken from Fig 3B, and in broken lines the lower limit absorption spectrum derived from it. Both curves have been given the

ordinate height by plotting extinctions on a percentage basis. The spectrum of pure rhodopsin lies within the limits set by these spectra.

The spectrum of a whole preparation from alum-treated retinas (from curve *a* of Fig. 6) is also shown in Fig. 9. If this be accepted as the upper limit of rhodopsin absorption—and there is no known reason to deny it this significance—then the spectrum of pure rhodopsin lies within the very narrow hatched area enclosed between this and the lower limit spectrum. One may with considerable confidence conclude from these observations that the extinction of pure rhodopsin

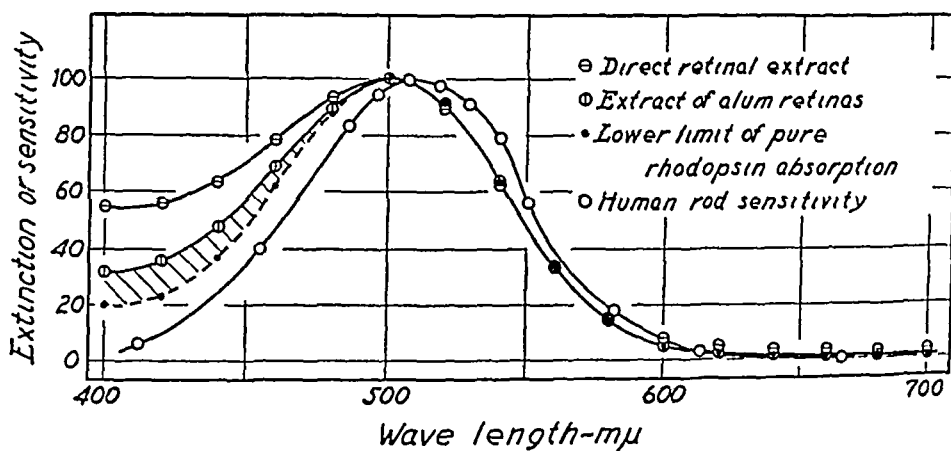


FIG. 9 Limits of absorption of pure rhodopsin, and human rod visibility. The latter function is from Hecht and Williams (1922-23). The direct retinal extract spectrum is from curve *a*, Fig. 3 B. The broken line shows the lower limit of rhodopsin absorption derived from it as described in the text. Between these curves is drawn the direct spectrum of an extract of alum-treated retinas, from curve *a* of Fig. 6. The spectrum of pure rhodopsin lies within the limits of the hatched area.

at 400  $m\mu$  lies between 0.20 and 0.32 of that at 500  $m\mu$ , and this exclusive of any absorption or scattering of light due to the protein residue of the rhodopsin molecule.

## VI

### Concentrations

Extracts of rhodopsin in the present experiments, though not complete, contained reasonably reproducible quantities of pigment. Extinctions of extracts of alum-treated retinas from bullfrogs, rabbits,

and rats are shown in Table I. These data were selected for maximal purity of the preparations, as evidenced by the absorption spectra. Extracts from non alum treated tissues were more variable, but contained about the same average quantities of pigment.

The third column of Table I presents the average extinction at 500  $m\mu$  of the total extract from one retina, concentrated in 1 cc, and

TABLE I  
*Extinctions at 500  $m\mu$  of Extracted Rhodopsin*

Column III shows the extinction per retina, for the total extract in 1 cc, measured in a 1 cm layer. By dividing the averages in this column by the retinal areas (column IV), the extinction of the total extract spread evenly over the retinal surface is obtained (column V). In column VI these values are reduced to percentages absorbed.

I	II	III	IV	V	VI
Animal	No. of retinas	Extinction per retina 1 cc. 1 cm.	Retinal area	Extinction per retinal surface	Absorption per retinal surface
			sq cm		per cent
Bullfrog	6	0.440			
	6	0.604			
	6	0.468			
	6	0.380			
	8	0.456			
	6	0.496			
Averages		0.472	2.65	0.178	33.6
Rabbit	8	0.116			
	8	0.104			
Averages		0.110	5.65	0.019	4.2
Rat	11	0.040	0.66	0.061	13.0

measured in a layer 1 cm in depth. Expressed differently, this is the extinction of extracted rhodopsin per retina, spread uniformly over a surface 1 sq cm in area. These figures provide no relative measure of retinal concentrations until corrected with the appropriate retinal areas.

In the fourth column of the table the average areas of the retinas

are shown. They were obtained by measuring the diameter of each type of fundus and computing area as though the retina were spread over the surface of a hemisphere of this diameter.

By dividing the extinction per retina at area 1 sq. cm. by the retinal area, one obtains the values shown in the fifth column. These are the extinctions of extracted pigment per retina, as though spread in a uniform layer over the retinal surface. They are re-stated as percentage absorption in the sixth column. They show the bullfrog retina to possess about three times as great a density of rhodopsin as the rat retina, and this in turn about three times that of the rabbit retina.

These values constitute the lower limit which may be assigned to the extinction of rhodopsin *in situ*. The true extinction *in situ* is certainly higher, since the extractions were not quantitative, and since rhodopsin is not uniformly distributed in the retina, but is concentrated at discrete points, the outer limbs of the rods. This latter factor introduces a much greater discrepancy in the rabbit retina, which is considerably diluted with cones, than in the rat, which possesses very nearly a pure rod retina (Lashley, 1932, Walls, 1934).

## VII

### DISCUSSION

*Rhodopsin Absorption and Rod Visibility* —The absorption spectrum of rhodopsin has been believed to resemble almost exactly the effectiveness of monochromatic lights in bleaching this pigment (Trendelenburg, 1904) and in stimulating the rods (König, 1894, Hecht and Williams, 1922-23).

This comparison has been based, not upon the absorption spectrum, but on the "difference spectrum" of rhodopsin. As Fig. 9 shows, the absorption spectrum of pure rhodopsin differs in form and position from the rod visibility curve.

A second consideration, however, negates these differences, and restores the original conclusion. The original comparison involved not only a mistaken absorption spectrum, but apparently also an inappropriate visibility function.

Hecht and Williams have accurately determined the rod visibility in a group of forty-eight observers of average age 25 years. Their



data are reproduced in the first two columns of Table II, and in Fig 9 They show the visibility as reciprocal of relative energy incident on the corneal surface of the eye, required to stimulate a constant sensation of brightness

TABLE II

*Rod Visibility and the Limits of Absorption of Pure Rhodopsin*

Columns I and II are from Hecht and Williams (1922-23) Column III is the dividend of column II by I Column IV is from Roggenbau and Wetthauer (1927) Column V is the dividend of III by IV Column VII is computed from Fig 6 a, column VIII from Fig 9, as explained in the text All visibilities and absorptions have been reduced to a common percentage basis for comparison

I	II	III	IV	V	VI	VII	VIII
Wave length	Energy visibility at cornea	Quantum visibility at cornea	Transmission of ocular media	Quantum visibility at retina	Wave length	Rhodopsin absorption (upper limit)	Rhodopsin absorption (lower limit)
mμ			per cent		mμ	per cent	per cent
412	6.32	7.82	19.3	18.3	400	35.2	22.0
455	39.95	44.7	33.0	61.4	420	39.4	23.5
486	83.40	87.5	40.5	94.2	440	51.9	39.4
496	93.90	96.5	43.8	100	460	72.5	64.3
507	99.35	100	45.7	99.0	480	90.8	88.3
518	97.30	95.8	47.8	90.9	500	100	100
529	91.10	87.8	49.9	79.9	520	91.7	91.7
540	78.78	74.4	51.0	65.7	540	66.3	67.2
550	55.60	51.5	52.8	43.4	560	37.3	36.5
582	17.78	15.6	57.5	12.3	580	15.8	14.9
613	2.72	2.26	58.7	1.75	600	5.8	5.9
666	0.181	0.138	66.5	0.093	620	3.3	3.3
					640	2.1	2.1
					660	1.7	1.7
					680	1.2	1.2
					700	0.8	0.8

The incident energy is absorbed by rhodopsin in discrete quanta, which vary in size inversely with the wave length, but are very likely equivalent in their effects To appreciate its action on rhodopsin, incident energy should be converted into relative numbers of incident quanta This is done by multiplying the energies by the respective wave lengths, or alternatively by dividing the respective visibilities by the wave lengths The results are shown in column III of Table II

The change from energy visibility to quantum visibility is very small, but in the direction of better fit with the absorption data (Dartnall and Goodeve, 1937)

To this point the visibility data deal only with the numbers of quanta incident on the corneal surface. For comparison with their effects on the retinal rhodopsin it is necessary to know the proportions of incident quanta at each wave length which penetrate to the outer limbs of the rods.

It is commonly stated in the ophthalmological literature that the transmission of human ocular media is practically complete throughout the visible spectrum. This statement is based upon very rough qualitative observations. Recent exact measurements show that actually the ocular tissues absorb a large fraction of the incident light, particularly at low wave lengths. Roggenbau and Wetthauer (1927), in a careful examination of the transmission of cornea, vitreous, and lens in cattle, have shown that at 400  $m\mu$  only about 16 per cent of the incident energy is transmitted to the retinal surface, while at 500  $m\mu$  the transmission has risen only to about 44 per cent. Ludvigh and McCarthy at the Howe Laboratories of Ophthalmology in Boston have recently determined the transmission of human ocular tissues in the visible. These data are not yet published, and have not been available to me. However, Dr Ludvigh has kindly informed me that the human data for an age group averaging 22 years resemble closely the data of Roggenbau and Wetthauer without correction. In younger human eyes the average transmissions are higher, in older eyes lower.

One may therefore, as a first approximation, use the Roggenbau and Wetthauer transmission data to correct the Hecht and Williams visibility function. This is almost certainly in part an under-correction, since the transmission data go only to the retinal inner surface, and so do not include absorption of light by the retinal tissues which screen the outer limbs of the rods. Particularly in the mammalian retina, with its rich network of capillaries, this factor might involve a large additional absorption of light.

Transmission of light to the retinal surface, taken from the data of Roggenbau and Wetthauer, is presented in column IV of Table II. The numbers in column III, divided by these factors, yield the "ret-

inal visibility," the reciprocals of relative numbers of quanta incident on the surface of the retina, required to stimulate a constant brightness sensation. These are shown in column V, and in Fig. 10.

It may be noted that it is only the retinal visibility, ideally the reciprocals of numbers of quanta incident on the outer limbs of the rods, that should possess a unique form. Any more peripheral type of visibility curve should vary with the individual transmission char-

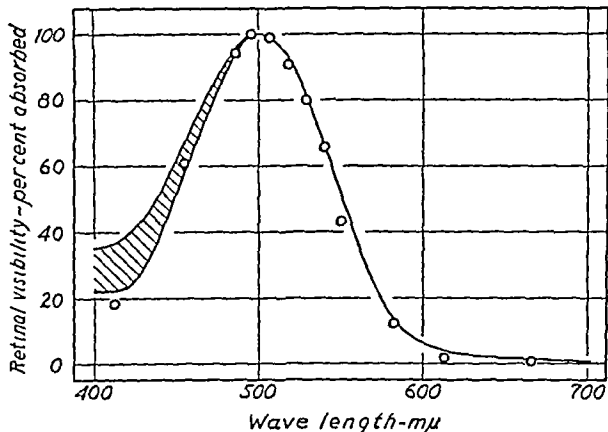


FIG. 10 Human rod visibility at the inner retinal surface (circles), and the absorption limits of pure rhodopsin *in situ* (hatched area), computed as described in the text. To facilitate comparison, these functions are plotted as percentages of their respective maxima.

acteristics of the observer's eye. Such variation has been clearly demonstrated in Abney and Watson's measurements of "external" rod visibility in various retinal areas of eight observers (1915). In fields  $10^\circ$  above the fovea, for example, the wave length of maximum visibility varied from about 495 mμ in the case of a student, Ar, to about 515 mμ in Abney himself, and the visibilities at 425 mμ in these two instances varied from 13 to 5.6 per cent of those at the re-

spective maxima A thoroughly corrected retinal visibility function should be absolute and invariant, but computation of such a relation must await the collection of visibility and ocular transmission data in statistically significant quantities

The retinal visibility is to be compared with the absorption spectrum of pure rhodopsin in solution Hecht has stated the theoretical basis for the belief that visibility varies proportionately with the *percentage absorption* (Hecht and Williams, 1922-23) <sup>6</sup> Though the form of an extinction spectrum is independent of concentration and depth of the absorbing layer—since the extinction is directly proportional to both these parameters—the shape of a percentage absorption spectrum is specific for one concentration and depth of layer For accurate comparison it is necessary, therefore, to estimate the true percentage absorption of rhodopsin *in situ* in the retina under consideration

It was shown above (Table I) that the extinction at 500 m $\mu$  of rhodopsin extracted from rat retinas, computed as though spread in a homogeneous layer over the retinal surface, is 0.061, and it was indicated that the extinction of rhodopsin in the rod outer limbs is surely considerably greater We may estimate roughly the extinction *in situ* as double this value, or about 0.12 <sup>7</sup>

<sup>6</sup> It is assumed that to produce a constant visual effect, a constant quantity of light—or more accurately a constant number of quanta—must be absorbed If  $I$  is the incident intensity at any wave length, and  $\alpha$  the percentage absorption, then for constant visual effect,  $\alpha I = \text{constant}$  But  $1/I$  for constant visual effect is the visibility Hence the latter is directly proportional to  $\alpha$  Recently Hecht (1937) has pointed out that  $\alpha$  is related to  $\epsilon$ , the absorption coefficient ( $= 2.303 \times$  extinction) by an expansion of the form,  $\alpha = \epsilon - \frac{\epsilon^2}{2!} + \frac{\epsilon^3}{3!}$  For small values

of  $\epsilon$  all but the first member of this series may be neglected and  $\epsilon$ —or extinction—substituted directly for  $\alpha$  in comparisons with the visibility In the present computations, however, this substitution entails considerable error I have preferred, therefore, to use the older, precise relation

<sup>7</sup> I have chosen the rat retina for this estimate, as a structure in which the rods approximate those of the human retina and are almost undiluted with cones Dartnall and Goodeve (1937), starting with data of frog extractions, have arrived at 0.1 as a probable extinction of rhodopsin *in situ* in the human eye The close correspondence between their estimate and mine is fortuitous As these authors properly indicate, doubling this estimate would not appreciably affect the argument

The limits of rhodopsin absorption, shown as the hatched area in Fig 9, have therefore been re computed with extinctions at 500  $m\mu$  set at 0.12. They have then been converted into percentages absorbed, and the maxima arbitrarily set at 100 for purposes of comparison. They are presented in this form in the last three columns of Table II and in Fig 10.

It is clear that the limits here assigned for rhodopsin absorption *in situ* agree very closely with the provisional retinal visibility. The small departure of the point at 412  $m\mu$  is scarcely to be regarded seriously. As already indicated, the visibility function is almost certainly under corrected, and all of the low wave length points should probably be raised. Within the limitations of available information, therefore, it may be concluded that the retinal visibility agrees in both form and position with the absorption spectrum of pure rhodopsin.

*Kinetics*—The present results show the complete process of bleaching of rhodopsin in solution to be complicated beyond any present hope of useful kinetic analysis. It should be possible, however, to regulate bleaching so that its over all velocity is limited by the speed of the photoprocess alone. In this way the kinetics of the isolated light reaction might be explored.

Chase (1935-36), using irradiation intensities much lower than those of the present research, found the kinetics of bleaching in acid and cold solutions to present "anomalies," while in warmer and more alkaline solutions the results permitted simpler interpretation. Chase ascribed these differences to the appearance of yellow reaction products under the former conditions, their absence under the latter. As shown above, new yellow products actually appear in all known circumstances.

The conditions for precise measurement of the kinetics of the photoprocess may be stated as follows:

1. The velocity of the light reaction must limit that of the entire transformation. This is accomplished by decreasing its speed by lowering the irradiation intensity, while simultaneously hastening the thermal components by raising the temperature and alkalinity. These conditions ensure that at all stages of bleaching only rhodopsin and the final yellow product (retinene protein) are present in the reaction mixture.

2. The concentration of rhodopsin in this mixture must be meas-

ured Obviously this is not given directly by the extinction at any wave length at which the yellow product itself absorbs light It is clear from Fig 8 that this prohibition involves appreciably all wave lengths below about 580  $m\mu$  in neutral or acid solutions, and below about 500  $m\mu$  in alkaline solutions The error introduced in this way in measurements of decrease in extinctions of neutral solutions at 500  $m\mu$  is of the order of 10 to 15 per cent Trendelenburg's measurements of bleaching in presumably neutral solution at 589  $m\mu$ , and Chase's measurements at 500  $m\mu$  and pH 9.3, are probably the only existent accurate spectrophotometric descriptions of the photoprocess

Hecht's detailed measurements of rhodopsin bleaching kinetics still remain the most acceptable (1920-21, 1923-24) Hecht principally employed low intensities of irradiation at room temperatures, so that bleaching occupied from 1 to 2 hours Under these conditions the speed of the light reaction almost certainly limits the velocity of bleaching Concentrations were measured by the rough but otherwise unexceptionable method of visual comparison with standard mixtures of rhodopsin and the final yellow product In a number of Hecht's experiments in which relatively high intensities and low temperatures were employed, the light reaction should not have limited the velocity of bleaching Yet such experiments did not yield anomalous results Apparently Hecht's colorimetric method succeeds in estimating the rhodopsin content of bleached solutions comparatively undisturbed by the state of the bleached products It is curious that this superficially rough procedure seems much better suited to the purpose than the more elegant spectrophotometric methods since employed There is no present reason for doubting Hecht's results They show that the photoprocess is first order, and that its velocity is directly proportional to the light intensity, and is practically independent of the temperature between 6.1 and 36.1°C

*Bleaching and Denaturation*—Most of the available evidence that rhodopsin is a protein involves its sensitivity to common protein denaturants (Wald, 1935-36) Recently Mirsky (1936) has suggested that the bleaching of rhodopsin by light is a denaturation process

The term denaturation includes a number of diverse changes primarily the loss of solubility at the isoelectric point, and associated

with this in varying degree a loss of specificity, or of ability to crystallize or to spread on a surface, increase in the number of exposed reducing groups, and changes in acid base properties (Neurath, 1936, Mirsky and Pauling, 1936, Bull and Neurath, 1937) None of these changes has yet been shown to accompany the bleaching of rhodopsin by light Unless the definition of denaturation is expanded to include all changes in the prosthetic groups of conjugated proteins, I see no present reason for calling the photodecomposition of rhodopsin a denaturation

It seems to me that denaturation of rhodopsin may occur with bleaching, or without bleaching, or that bleaching may occur without denaturation, depending upon what is done to the molecule Strong acids and alkalis, alcohol, chloroform, acetone in the warm, and temperatures above 60°C all convert rhodopsin irreversibly to yellow with denaturation of the protein residue It may be that careful experimentation will eventually show some of these effects to be reversible The behavior of the related carotenoid protein, *ovoverdin*, foreshadows what may be expected of rhodopsin in this regard (Stern and Salomon, 1938)

On the other hand the protein residue of rhodopsin may be profoundly altered without a trace of bleaching, as when retinas are treated for long periods with 8 per cent formol, and probably also when they are soaked for days in 4 per cent alum, or are allowed to rot intensively at 40°C (Ayres, 1882)

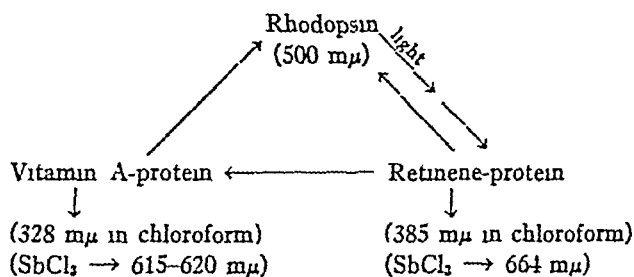
Finally, the prosthetic group of rhodopsin may be attacked directly by absorption of light That in this case the protein residue is appreciably affected, apart from changes in the carotenoid protein bond, is still to be demonstrated

There is a particularly interesting situation in which denaturation processes may cooperate with photoprocesses to produce bleaching This appears among the observations of Ewald and Kühne (1878)<sup>\*</sup> Below about 37°C bleaching is typically photochemical, it occurs only in light, and its velocity is practically independent of the temperature (see also Hecht, 1920-21) Above about 50°C bleaching occurs by a typical denaturation process, in darkness, and possesses a very high temperature coefficient In the intermediate range from

<sup>\*</sup> Ewald and Kühne, 1878 IV, 440

40 to 50°C, bleaching requires light, but possesses also a high temperature coefficient. It is possible that within this range the increase in internal energy of rhodopsin molecules effectively raises the quantum efficiency.

*The Visual Cycle*—The final product of bleaching of rhodopsin in solution is a mixture of retinene and protein, in part loosely bound to each other. In the isolated retina, two reactions are added to the system *in vitro*: (1) more pronounced and regular reversion of retinene to rhodopsin, and (2) conversion of retinene to vitamin A. Finally, in the intact eye, the visual cycle is completed with reversion of vitamin A to rhodopsin (Wald, 1935-36, 1936-37). The entire cycle is expressed conveniently in the following skeletal form:



The bleaching of rhodopsin to retinene-protein has now been shown to proceed by a complex succession of photic and thermal processes. Other reactions in the retinal cycle may be similarly complicated. The arrangement of apparent precursors as written here is also not to be interpreted rigidly. Now that retinene is recognized to be only the last of a series of products of bleaching, it is clear that any of the intermediates might be the true precursor for formation of either rhodopsin or vitamin A.

It is noteworthy in this connection that the color of retinas in all stages of bleaching, following exposure to bright light, is orange, not yellow, and resembles therefore the color of the initial photoproducts rather than that of retinene. This distinction is apparently not simply a physical effect, for on strongly acidifying an orange retina the strikingly different yellow color of acidic retinene at once appears (Wald, 1936-37). It is quite possible, therefore, that the color of irradiated retinas is due principally to the initial photoproducts. Certainly retinene is present in the retina, since it may be extracted di-



rectly with benzene, but perhaps normally it occurs in low concentration. We may imagine retinene to be in mobile equilibrium in the irradiated retina with orange photoproducts, extraction displaces the equilibrium and removes all the pigment in the form of retinene. On the other hand, in solutions of irradiated rhodopsin, retinene accumulates because it is formed irreversibly. From this point of view retinene may occupy much the same position in the retinal cycle that hexose diphosphate does in muscle—that of an intermediate normally present in very small amounts, but capable of accumulating in large quantity when the normal system is reduced by poisoning of the tissue or by extraction (Meyerhof, 1937).

These are matters for further experimentation to decide. For the present the cyclic scheme presented above expresses correctly certain relations among the three most stable loci in the complex retinal cycle. It summarizes simply a large number of the empirical observations. It has already proved useful in describing the behavior of the functioning visual system (Winsor and Clark, 1936, Riggs, 1937, Hecht, Haig, and Chase, 1936–37, Wald and Clark, 1937–38).

#### SUMMARY

1 The properties of rhodopsin in solution have been examined in preparations from marine fishes, frogs, and mammals.

2 The bleaching of neutral rhodopsin in solution includes a photic and at least three thermal ("dark") processes. Thermal reactions account for approximately half the total fall in extinction at 500  $m\mu$ .

3 Bleaching has been investigated at various pH's from 3.9 to about 11. With increase in pH the velocity of the thermal components increases rapidly. Though the spectrum of rhodopsin itself is scarcely affected by change in pH, the spectra of all product mixtures following irradiation are highly pH labile.

4 The spectrum of pure rhodopsin—or of the rhodopsin chromophore—is fixed within narrow limits. The extinction at 400  $m\mu$  lies between 0.20 to 0.32 of that at the maximum.

5 Within the limitations of available data, the spectrum of pure rhodopsin corresponds in form and position with the spectral sensitivity of human rod vision, computed *at the retinal surface*.

6 The nature of bleaching of rhodopsin in solution, its kinetics, and its significance in the retinal cycle are discussed.

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# THE EFFECT OF HYDROGEN ION CONCENTRATION UPON THE INDUCTION OF POLARITY IN FUCUS EGGS

## III GRADIENTS OF HYDROGEN ION CONCENTRATION\*

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### INTRODUCTION

It has been shown (Whitaker (1)) that the tendency of the *Fucus* egg to form a rhizoid on the side toward a neighbor, or in the resultant direction of neighbors ("group effect"), is greatly intensified or augmented when the sea water medium is acidified to pH 6.0. When single eggs are placed near one end in a close fitting tube of glass or quartz 10 or 20 egg diameters long so that substances diffusing from the egg escape more rapidly from one side than from the other, the egg develops in a gradient of its own diffusion products. It has been shown (Whitaker and Lowrance (2)) that in this case also acidification of the medium increases the tendency of the egg to form the rhizoid on the side of greatest concentration of substances diffusing from the egg. The polarity of the egg is determined by the concentration of substances diffusing from the egg itself, just as it may be determined by concentration gradients brought about by a neighboring egg.

A general or uniform increase in the concentration of hydrogen ions, which intensifies the response of the egg to gradients, should not be confused with gradients of concentration of hydrogen ions or of other substances across the developing eggs. Groups of eggs or even two eggs alone in a dish developing in close proximity in the dark, or single eggs near one end in glass tubes undoubtedly produce pH gradients across the developing eggs as a result of the diffusion patterns of CO<sub>2</sub> and perhaps other acid metabolites. However, these pH gradients,

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which may be presumed to exist under conditions which result in the group effect, coincide with concentration gradients of any and all substances diffusing into or from each egg cell. In order to separate the effects of hydrogen ions from the effects of other substances except  $\text{CO}_2$  (see discussion), the effect of pH gradients has been tested directly in the experiments now to be reported. A preliminary report of a part of the results has been published (3).

### Method

Ripe fruiting tips of *Fucus furcatus f. luxurians* were collected at the same locality and by methods which have previously been described (4). The fruiting tips were kept for a few days at  $4^\circ\text{C}$ . When gametes were obtained they were kept in the dark except for brief exposure during parts of the experiment to red light, which does not affect the polarity of *Fucus* eggs. All work was carried out in a constant temperature room at  $15 \pm \frac{1}{4}^\circ\text{C}$ , and no eggs were used which did not appear to be spherical at the start. Material was collected and the experiments were carried out in March, April, May, and June, 1935.

Diffusion pipettes were made of Pyrex glass. Each pipette was drawn with a very steep taper from 10 mm stock. The finished pipette consisted of a tube of Pyrex 10 mm in diameter and about 5 cm long, but at one end within approximately 1-2 cm of length the tube was tapered to an overall diameter of about 25 microns, and a lumen of about 8-20 microns diameter. The tapers were drawn at an angle of about  $45^\circ$  with respect to the long axis of the primary tube. Pipettes of similar size and shape were matched in pairs and the tips were cut off so that the lumens were of similar diameter. The tips were cut off with a diamond mounted in a Taylor micromanipulator.

The pipettes were filled from the tips to the bases of the tapers with 1 per cent agar-sea water so that the main body of the pipette could be refilled with liquid without leaving an air bubble between the liquid and the inner surface of the agar. Two matched pipettes were used in each experiment. One of the pipettes was filled with normal sea water and the other was filled with acidified sea water. To equilibrate the agar in the tip with the solution to be used in the experiment each pipette was filled and refilled several times, and the tip was also soaked in the same solution. The level of the solution in the pipette was several centimeters above the solution in which the tip was maintained so that some pressure supported the diffusion. The agar in the tips of the pipettes was equilibrated over a period of 11-16 hours before the start of an experiment.

In each experiment a single egg was reared in a pH gradient. The egg was placed in a square Petri dish of normal sea water on the levelled stage of a microscope on a concrete table. These precautions were necessary to keep the egg from moving. Sea water from the same supply was used in the Petri dish and in the normal sea water pipette. The two pipettes were freshly filled with fluid

to the same level which ranged from 3 to 15 mm above the level of the sea water in which the egg was placed, so that the diffusion was supported by some pressure. The agar plugs apparently adhered firmly to the glass and no detectable volume of fluid passed from the pipettes into the culture dishes during the experiment. About 2 hours after fertilization the two pipettes, which were mounted in a Taylor micromanipulator at an inclined angle so that the finely drawn tapers were approximately horizontal, were approached toward the egg on opposite sides until the tips were level with the egg and were about an egg diameter from it. Fig 1 shows photomicrographs of the eggs and pipette tips at a later stage of development. The two tips were originally equidistant from the spherical egg, and this relation usually persisted although in some cases the egg moved slightly at some time during development. In earlier experiments the egg rested on the glass bottom of the dish. In later experiments a level layer of sea water agar was placed in the bottom of the dish to reduce breakage of pipettes at the time of set up. Four micromanipulators were available and four experiments were run simultaneously.

The normal sea water pipette provided a control for the glass, agar, etc. of the pipette. The need for this control was suggested by collateral observations that *Fucus* eggs may be affected by a nearby glass surface probably because of its interference with diffusion of products from the eggs.

In selecting an agent for acidifying the sea water in the acid pipette it was regarded as desirable to have enough buffer capacity to be sure that the acid diffusing from the very small lumen of the pipette would not too readily be neutralized by the sea water. On the other hand, it would be undesirable to have so much buffer capacity that acidity would be maintained in high degree when the buffer diffused around to the other side of the egg. From these considerations, a mixture of 4 or 5 parts of McIlvaine's<sup>1</sup> buffer to 96 or 95 parts of sea water was selected. As pointed out in the first paper of this series (Whitaker (1)), such mixtures provide enough buffer capacity to hold the pH constant for 24 hours to within 0.1 pH unit when eggs are growing in them, but they contain the least amount of buffer which will do so. Upon dilution with sea water the buffer capacity and the hydrogen ion concentration decrease rapidly. Since the mixture diffusing from the acid pipette would be very greatly diluted before reaching the far side of the egg these conditions should be favorable to an effective pH gradient.

After definite and convincing results had been obtained, it was thought desirable to carry out a few more experiments using another buffer system involving neither citrate nor phosphate to be sure that the results could not be attributed to these ions. For this purpose acidification was carried out by means of a mixture of HCl and NaHCO<sub>3</sub> added in such amount as to give a buffer capacity which was empirically found to be approximately the same as the buffer capacity of the

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<sup>1</sup> A mixture of 0.1 M citric acid and 0.2 M secondary sodium phosphate in such proportions as to give the desired pH.

4 per cent or 5 per cent McIlvaine's mixture After acidification with either buffer system the sea water was equilibrated with atmospheric  $\text{CO}_2$ , and the osmotic pressure of the mixtures was readjusted if necessary with glass distilled water

TABLE I

The conditions of the experiments (see Figs 1, 2, and 3 for results) The pH of the sea water as given is the initial pH at the start of each experiment Under the heading "buffer", McIlv refers to McIlvaine's buffer See text

Experiment No	Sea water pH	Acid pipette		Experiment No	Sea water pH	Acid pipette		Experiment No	Sea water pH	Acid pipette	
		pH	Buffer			pH	Buffer			pH	Buffer
		per cent				per cent					
1	7.8	6.4	McIlv 4	22	8.0	6.0	McIlv 4	43	8.1	5.9	McIlv 4 per cent
2	7.7	6.2	" 4	23	7.5	6.0	" 5	44	7.7	6.2	HCl-NaHCO <sub>3</sub>
3	8.3	6.0	" 5	24	7.5	6.2	" 4	45	7.7	6.2	" "
4	7.5	6.0	McIlv 5	25	7.5	6.0	McIlv 5	46	7.6	6.1	HCl-NaHCO <sub>3</sub>
5	7.5	6.0	" 5	26	7.4	6.0	" 5	47	7.6	6.1	" "
6	7.5	6.2	" 4	27	7.4	6.0	" 5	48	7.6	6.1	" "
7	8.1	5.9	McIlv 4	28	7.7	6.2	McIlv 4	49	7.7	6.2	HCl-NaHCO <sub>3</sub>
8	8.1	5.9	" 4	29	7.5	6.0	" 5	50	7.7	6.2	" "
9	7.7	6.2	" 4	30	7.7	6.2	" 4	51	7.6	5.5	McIlv 4 per cent
10	8.3	5.8	McIlv 5	31	7.6	6.0	McIlv 4	52	7.6	5.5	McIlv 4 " "
11	7.8	6.4	" 4	32	8.3	5.8	" 5	53	7.6	5.6	" 4 " "
12	7.7	6.2	" 4	33	7.5	6.0	" 5	54	7.6	5.6	" 4 " "
13	7.8	6.4	McIlv 4	34	8.3	5.8	McIlv 5	55	7.6	5.6	McIlv 4 " "
14	8.1	5.9	" 4	35	7.4	6.0	" 5	56	7.6	5.5	" 4 " "
15	7.7	6.0	" 5	36	7.4	6.0	" 5	57	7.6	5.5	" 4 " "
16	7.5	6.2	McIlv 4	37	8.0	6.0	McIlv 4	58	7.6	5.6	McIlv 4 " "
17	7.7	6.0	" 5	38	8.1	5.9	" 4	59	7.8	5.7	HCl-NaHCO <sub>3</sub>
18	7.8	6.4	" 4	39	8.3	5.8	" 5	60	7.8	5.7	" "
19	7.5	6.0	McIlv 5	40	7.8	6.4	McIlv 4	61	7.8	5.7	HCl-NaHCO <sub>3</sub>
20	7.6	6.0	" 4	41	7.5	6.2	" 4	62	7.8	5.7	" "
21	8.1	5.9	" 4	42	8.3	6.0	" 5	—	—	—	—

## RESULTS

After setting up the pipettes, the dish containing the egg was covered to reduce evaporation, and the assembly was ordinarily left undisturbed for 24 hours or longer By this time the rhizoid protuberance was well developed and the first cell division was usually completed The results were sketched and photographed

Figs 1, 2, and 3 show photomicrographs of all eggs reared in pH



gradients, except that the result of experiment 7 (Fig. 1) is shown as a drawing from the original because the photograph failed. In all cases the right hand pipette in each boxed figure which is also the pipette under which the number of the experiment appears is the acid pipette. For convenience in considering the results shown in the figures the numbers of the experiments are given not in the order in which they were carried out, but in the order of the type of result. Table I gives the conditions of each experiment.

Fig. 1 shows all eggs which were reared with McIlvaine's buffer used as the acidifying agent in the acid pipette. The data in Table I show that the pH of the normal sea water at the start of these experiments (experiments 1-43) ranged from 7.4 to 8.3 and that the pH of the medium in the acid pipette ranged from 5.8 to 6.4. Of the 43 eggs 39 or 91 per cent formed rhizoids on the acid half of the egg and 21 or nearly half formed them within  $10^\circ$  of the most acid point of the egg surface.

7 more eggs were then reared with  $\text{HCl-NaHCO}_3$  used as the acidifying agent in the acid pipette, and the results are shown in Fig. 2. In these experiments (44-50) the initial pH at the tips of the pipettes ranged from 7.6 to 7.7 (basic) and from 6.1 to 6.2 (acid). All of the eggs formed rhizoids on the acid side. The results are thus essentially the same regardless of which buffer system is used. Considering experiments 1-50 together 46 eggs or 92 per cent formed the rhizoid on the acid side of the egg.

While most of the eggs formed rhizoids quite close to the most acid point of the egg surface (Figs. 1 and 2), a considerable number which formed them on the acid side did so at some distance from the most acid point. A number of considerations suggested that this might be due to slightly greater than optimum acidity at the most acid point of the egg surface resulting from greater acidity of the solutions, greater diameter of the pipette tips, closer proximity of the tips to the egg, or a combination of such factors. To test this, experiments 51 to 62 were arranged with still greater acidity of the acid pipette. The results are shown in Fig. 3.

In experiments 51-58 (Fig. 3) the medium in the acid pipette was acidified to pH 5.5-5.6 with McIlvaine's buffer. In experiments 59-62 it was acidified to pH 5.7 with  $\text{HCl-NaHCO}_3$ . The results

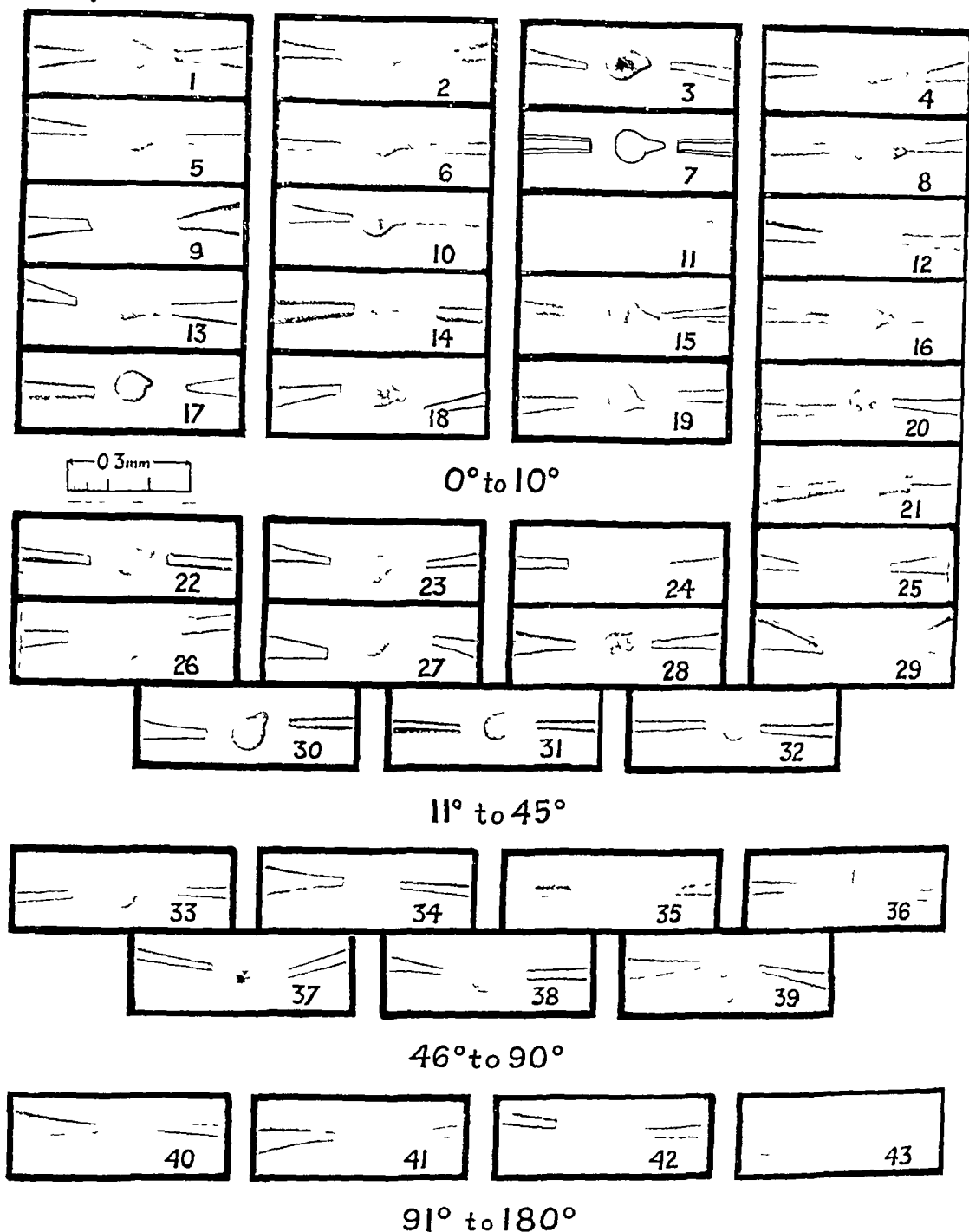


FIG 1 Photomicrographs of results of experiments in which an egg developed in a pH gradient and in which McIlvaine's buffer was used to acidify the sea water in the acid pipette. In each photograph the acid pipette is shown on the right hand side, above the number of the experiment. See Table I and text for conditions of each experiment. The result of experiment 7 is shown as a drawing.

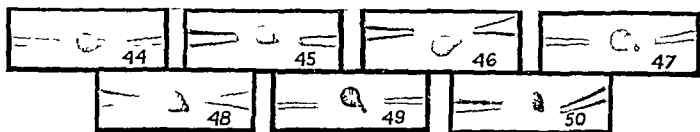


FIG 2 Photomicrographs of results of experiments in which an egg developed in a pH gradient and in which HCl/NaHCO<sub>3</sub> was used to acidify the sea water in the acid pipette. In each photograph the acid pipette is on the right hand side above the number of the experiment. See Table I and text for conditions of each experiment.

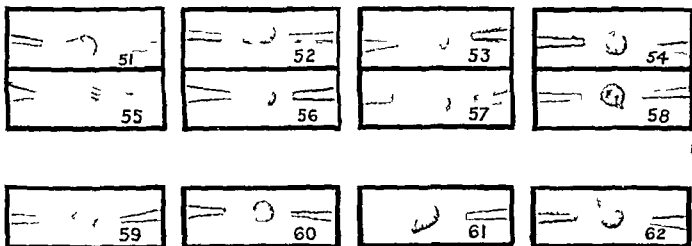


FIG 3 Photomicrographs of experiments in which an egg developed in a pH gradient and in which the sea water in the acid pipette (shown on the right hand side in each photograph above the number of the experiment) was acidified to pH 5.7-5.5. This is slightly more acid than in the cases shown in Figs 1 and 2. In experiments 51-58 inclusive McIlvaine's buffer was used to acidify and in experiments 59-62 inclusive a mixture of HCl and NaHCO<sub>3</sub> was used. See Table I and text for conditions of each experiment.

show that when the pH of the medium in the acid pipette is lowered to 5.5–5.7 the rhizoid forms on the side of the egg away from the acid pipette.

At the end of the experiments a small amount of almost transparent deposit was quite often observed attached to the tip of the acid pipette, extending out from it for about 5 to 20 microns. This material was not identified. It may have been a precipitate of buffer ingredients, or possibly a growth of microorganisms. It never formed on the basic pipette. This deposit was variable in amount, and it was entirely absent in quite a number of experiments with McIlvaine's buffer and in all experiments in which  $\text{HCl-NaHCO}_3$  was used to acidify. No correlation could be found between its presence and the result, and it is therefore regarded as inconsequential.

In four experiments the pipettes were not maintained in place until the end as in all other experiments, but instead they were withdrawn at 15 hours after fertilization. At this time no rhizoid protuberances or any other external indications of polarity had yet appeared. The rhizoid protuberances later formed near the regions of the egg surfaces which had formerly been most acid. So far as this evidence goes, therefore, it indicates that the pH gradient may determine the developmental polarity some time before the morphological response takes place.

#### DISCUSSION

The results appear to show quite conclusively that the developmental polarity and the pattern of differentiation of the *Fucus* egg can be determined by a gradient of hydrogen ion concentration across the developing egg in sea water. Since the sea water contains carbonates, a diffusion gradient of hydrogen ion concentration may be expected to produce a gradient of  $\text{CO}_2$  tension by chemical action on the sea water. Even when all the excess  $\text{CO}_2$  resulting from acidification of the sea water is successfully removed by aeration from the fluid used in the acid pipette,  $\text{CO}_2$  would be produced again when the acid sea water diffused into more basic sea water. So long as a gradient of hydrogen ion concentration is maintained in carbonate-bearing sea water, a coincident gradient of  $\text{CO}_2$  tension is a corollary. The experiments therefore do not show whether the physiological

effects are due directly to the hydrogen ions or to the  $\text{CO}_2$  which they produce in the medium

It is not known whether an earlier polarity exists in the egg<sup>2</sup> which is superseded by the effect of the gradient, or whether polarity is first established under the influence of the gradient. The former is perhaps more probable, especially since an egg develops normally with no external gradient of any sort. In any event, any region of the cytoplasm may be caused to give rise to either rhizoid or thallus, depending on this external factor.

The rhizoid tends to form at or near the most acid point on the surface of the egg unless this is too acid, in which case it forms at a more basic region. In extreme cases it forms at the most basic point on the egg surface. Since eggs will develop rhizoids in medium buffered at pH 5.5 (1), although with some delay, it cannot be supposed that rhizoid formation is impossible at the absolute pH of the most acid point of the egg surface under conditions resulting in reversal. The reversals were obtained when the acid pipette was at pH 5.5-5.7. The rhizoid forms at a more basic point on the egg surface when one is available. It is clear that what happens at a given region of the egg depends not only on the pH at that region but also on the pH at other regions of the egg surface. In other words, it depends on the gradient as well as upon the absolute pH. This is in harmony with Child's theory of physiological gradients (6 and 7). The specific mechanism by which the gradient of hydrogen ion (or  $\text{CO}_2$ ) concentration determines the morphogenesis remains as the principal problem. A *Fucus* egg which apparently is not subjected to any external pH gradient develops normally and forms one rhizoid over a wide pH range. The differentiation therefore appears to be controlled by an internal system which is readily altered or shifted by a number of external factors, including the pH gradient.

The actual pH values at the egg surface which result in the particular responses are of course not known precisely. The effect of metabolites on the pH at the surface can only be guessed at, and the acid diffuses around the egg to acidify the basic side to an unknown extent. There is no doubt, however, about the relative pH values and the

<sup>2</sup> Knapp (5) reports that the rhizoid normally forms at the entrance point of the sperm in *Cystosira*.

directions of the pH gradients. Even when the pH of the solutions is reproduced there is, of course, a considerable variation in the pH effect of the acid pipette depending on its diameter, distance from the egg, steepness of taper, etc. Since reversal of the developmental response takes place when the pH of the acid pipette is lowered only slightly (from 5.9 to 5.7), the variation seen in the position of the rhizoids with respect to the most acid point, even when the pH of the pipettes is reproduced, may be due in considerable part to slight differences in the effectiveness of the pipettes. Some differences in the eggs are also to be expected, and possibly there is some residual effect of an earlier polarity.

The group effect phenomenon (see introduction) and the response of an egg in a capillary to its own diffusion products (2) can be accounted for adequately on the basis of acid metabolites, acting through diffusion patterns of hydrogen ions or  $\text{CO}_2$ . It has been pointed out earlier (1 and 2) that there are reasons for suspecting that a growth substance may be involved in the responses of the *Fucus* egg and that a number of agents which determine polarity may act by affecting growth substance. Since the known growth substances are acids which are physiologically active in the undissociated molecular form, hydrogen ions increase the activity of growth substance (8-10). Growth substance (auxin) softens cellulose walls, and it has further been shown by Thimann and Went and others (9) that auxin initiates and induces root differentiation. The rhizoid formation in *Fucus* involves softening of the cellulose wall, and at least by analogy the differentiation resembles root differentiation.

Recently du Buy and Olson (11) have succeeded in extracting an unidentified growth substance from eggs of *F. vesiculosus* with chloroform. Dr. du Buy informs me that the volume of the agar gel blocks which they used in the oat test was  $8 \text{ mm}^3$ . They obtained positive results using test blocks containing egg extract in a concentration approximately equivalent to 0.85 cc eggs per cc agar gel. In January, 1936, Drs. Went, Skoog, and van Overbeek, at the California Institute of Technology, were so kind as to test extracts which Dr. Lowrance and I made from recently fertilized eggs of *F. furcatus*. The tests were made with  $4 \text{ mm}^3$  agar blocks on seeded and de-seeded oat seedlings. Our extracts were made with purified chloroform, and

separate extractions were repeated on the same material with chloroform and HCl. The products of the primary neutral extractions were tested in equivalent concentrations of approximately 0.38, 2.4, and 3.8 cc eggs per cc agar gel. The acid extractions were tested in equivalent concentrations of approximately 0.9, 2.4, and 3.8 cc eggs per cc agar gel. The results were negative, and in May, 1936, Dr. van Overbeek tested some more extracts which we prepared at the Hopkins Marine Station from eggs reared in light (40 watts at 1 meter) until just before and just after rhizoid protuberances began to form. Chloroform partition extracts from the sea water medium were also added in. These extracts were tested in a similar manner. The concentrations of the products of neutral extraction were approximately equivalent to 0.4 and 1.7 cc eggs per cc agar gel, and the concentration of the products of a second acid extraction was 1.7. Again the results were negative, but negative results do not mean much in a case of this sort as special procedures are often necessary for the extraction of growth substance. It is also possible that oxidative destruction took place during the 30 to 48 hours required to transport the iced extracts to Pasadena and start the tests.

Olson and du Buy (12) recently report that rhizoids tend to form on the sides of developing eggs of *F. vesiculosus* to which the potassium salt of indole acetic acid (hetero auxin) is applied in sufficient concentration. As they point out, their results strengthen the hypothesis that growth substance in the egg induces rhizoid formation and that a number of agents which determine polarity may act by affecting the activity of growth substance. Most of the effects of the pH gradient can be interpreted on this basis very well. To explain the formation of the rhizoid on the basic side of the egg when the pH of the acid pipette is lowered to pH 5.7-5.5, it would probably be necessary to suppose that in this case so much growth substance is present in the molecular form at the most acid point that it is inhibitory. It may instead be that other effects of the hydrogen ions predominate at this concentration. It is more difficult to explain the rôle of hydrogen ions in terms of their effect on growth substance in the case of the egg developing near one end in a capillary tube, which tends to form the rhizoid on the side away from its own diffusion products (which is also presumably the basic side) when the pH of the medium is initially

high, *i.e.*, 8.1–8.4 (2) It is probable that hydrogen ions or  $\text{CO}_2$  also affect the underlying rhizoid forming processes in other ways than through the pH effect on the activity of growth substance

The results reported in this paper show that hydrogen ions and  $\text{CO}_2$ , produced as metabolites, are adequate to explain the group effect They probably act, at least in part, through their effects on growth substance in the egg Whether the effect is supported in addition by the diffusion of growth substance from neighboring eggs to form effective concentration gradients in the medium is undetermined

#### SUMMARY AND CONCLUSIONS

1 Gradients of hydrogen ion concentration across *Fucus* eggs growing in sea water determine the developmental polarity of the embryo

2 Gradients may determine polarity even if removed before the morphological response begins

3 The rhizoid forms on the acid side of the egg unless this is too acid, in which case it develops on the basic side of the egg

4 Since gradients of hydrogen ion concentration in sea water produce gradients of  $\text{CO}_2$  tension, as a result of chemical action on the carbonate buffer system, it is not proven whether the physiological effects are due to the hydrogen ions, or to the  $\text{CO}_2$  which they produce in the medium

5 The developmental response of the eggs to gradients of hydrogen ion (or  $\text{CO}_2$ ) concentration provides an adequate but not an exclusive explanation of the group effect in *Fucus*

6 Hydrogen ions may exert their effect by activating growth substance Hydrogen ions or  $\text{CO}_2$  probably also affect the underlying rhizoid forming processes in other ways as well

The author is indebted to Dr Edward Lowrance for assistance in carrying out the experiments

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*Relation of Phage Activity to the Protein*—If the nucleoprotein is really the active agent it would be expected that denaturation or digestion of the protein would result in corresponding loss in activity. The protein is instantly inactivated in solution more acid than pH 4.7. No visible precipitate forms, even in the presence of salt, but the solution becomes more viscous and the denatured protein may be filtered out. Measurement of the sedimentation constant shows the

TABLE VI  
*Inactivation and Denaturation at Various pH*

Noted amount M/1 acetic acid added to 5 ml phage solution in M/10 pH 7.0 phosphate buffer,  $\frac{1}{4}$  saturated ammonium sulfate. Solution at 20°C for 5 minutes, titrated back to pH 7.0 by 1 M ammonium hydroxide. 0°C for 24 hrs. Filtered at 0°C through No. 42 paper. Samples removed and analyzed for ph u and protein N as noted.

Ml M/1 acetic acid	0			0.7			1.0		
pH (colorimetric)	7.0			4.7			4.4		
	[ph u] ml	[protein N] ml	[ph u] mg pro tein N	[ph u] ml	[pro- tein N] ml	[ph u] mg pro tein N	[ph u] ml	[protein N] ml	[ph u] mg protein N
Solution analyzed immediately after neutralization,									
No. 1	500	0.08	6300	500	0.08	6300	1.0	0.08	1.0
After 24 hrs 0°C,									
No. 2	500	0.08	6300	500	0.08	6300	1.0	0.08	1.0
Solution No. 2, Filter No. 42	500	0.07	6600	420	0.06	7000	1.0	<0.001	>1000
2 ml solution No. 2 plus 4 ml $\frac{1}{4}$ saturated ammonium sulfate—viscosity measured 25°C									
$\frac{\eta_{sol}}{\eta_{H_2O}}$			1.3			1.3			1.55

denatured protein to be of much smaller molecular weight than the active protein.

The result of an experiment in which the protein was denatured by acid is shown in Table VI. At pH 7.0 and 4.7 there is no change in activity in 24 hours. At pH 4.4 practically all the activity is lost after 5 minutes at 20°C, the total protein content remains constant but the viscosity increases and after standing at 0°C for 24 hours the denatured, inactive protein may be filtered off.

*Inactivation and Denaturation at Various Temperatures*—The result of an experiment in which solutions at pH 7.0 were heated varying lengths of time at different temperatures is shown in Table VII. The loss in activity is again accompanied by the formation of denatured protein. It may be noted that the specific activity ([ph u]/mg protein N) in this experiment is low throughout and at 55°C tends to decrease. This indicates that the preparation already contained some denatured protein and also that the separation of native and

TABLE VII

*Inactivation and Denaturation of Phage Protein Solution pH 7.0 at Various Temperatures*

Solution in  $\mu/10$  pH 7.0 phosphate,  $\frac{1}{4}$  saturated ammonium sulfate left at temperatures shown and samples removed and cooled to 0°C for 24 hrs after time interval shown. Samples filtered through No. 42 paper at 0°C and activity and protein nitrogen determined on filtrate

Temperature.	55 C.			37 C.			25 C.			0 C.		
Time												
hrs	[ph u] ml	[protein N] ml	[ph u] mg protein N	[ph u] ml	[protein N] ml	mg protein N	[ph u] ml	[protein N] ml	mg protein N	[ph u] ml	[protein N] ml	mg protein N
0	20	0.01	2000	50	0.015	3500	50	0.015	3500	50	0.015	3500
0 016	10	0.007	1400									
0 03	5	0.005	1000									
0 16	0.1	0.001										
24 0				2	0.001	2000	15	0.005	3000			
72 0							5	0.001	5000			
120 0										20	0.007	3000
480 0										2	0.001	2000

denatured protein is not quantitative. This effect was also noted in analyzing known mixtures of native and denatured protein and renders this type of experiment less conclusive.

Krueger and Mundell (23) have shown that such heat inactivated phage may be reactivated under certain conditions. Lominski (24) has also reported similar results. This is probably a case of the reversal of denaturation of a protein and may furnish the explanation for Kendall's (25) experiments in which it was found that active phage may be obtained from autoclaved solutions.

TABLE VIII  
*Effect of Various Enzymes*  
 10 ml bacteriophage solution in N/10 phosphate, 0.1 saturated ammonium sulfate plus enzyme noted, analyzed for activity and protein nitrogen

Chymo trypsin																					
pH 7.0-7.4, 10°C																					
10 ml bacteriophage solution in N/100 protein precipitated by 10 ml 10% trypsin																					
Enzyme		0		Pepsin pH 2.0		Trypsin		Heat inactivated phage Trypsin		1.0		0.05		0.10		0.05		0.02			
Conc enzyme mg N/ml	days	[ph u] ml		[protein N] ml		[ph u] ml		[protein N] ml		[ph u] ml		[protein N] ml		[ph u] ml		[protein N] ml		[ph u] ml		[protein N] ml	
		150	0.036	—	0.036	150	0.040	—	0.032	1000	0.17	1000	0.17	1000	0.17	150	0.04	150	0.04	150	0.040
0	0	150	0.036	—	0.030	170	0.036	—	0.038	200	0.20	500	0.21	20	0.038	50	0.034	70	0.035		
1	1	150	0.036	—	0.030	120	0.032	—	0.040	0	0.17	200	0.19	1	0.030	—	—	—	—		
2	2	150	0.036	—	0.038	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
4	4	150	0.038	—	0.038	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
8	8	150	0.038	—	0.038	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
15	15	150	0.038	—	0.038	—	—	—	—	—	—	—	—	—	—	—	—	—	—		



*Hydrolysis of Phage by Various Enzymes*—The literature concerning the digestion or inactivation of phage by enzymes, especially "trypsin," is controversial. This is probably due to the fact that crude trypsin preparations, which contain several enzymes, have been used. It was found in the present work that phage was inactivated by chymo-trypsin (26) but not by trypsin. The denatured protein is not hydrolyzed by either pepsin, trypsin, or chymo trypsin. The effect of these enzymes on active and inactive phage preparations is shown in Table VIII. It will be noted that there is no significant change in the concentration of total protein nitrogen. There is, however, a qualitative change in the protein when the activity is destroyed by chymo trypsin. After inactivation the protein has the same properties as protein denatured by heat and may be removed by filtration or centrifugation. There is in this case also an increase in viscosity and concentrated solutions set to a gel. This action of chymo trypsin on the bacteriophage therefore appears to be very similar to its action on casein (26). Dilute chymo trypsin causes milk or casein solutions to clot without any marked change in total protein concentration although more concentrated enzyme solutions do digest the protein. The concentration of chymo trypsin required to inactivate phage solutions is very much higher than that required to clot milk and it is probable that still higher concentrations would hydrolyze the protein but the accurate determination of phage protein in the presence of such large amounts of chymo trypsin is experimentally difficult.

The results of a series of experiments in which the loss in phage activity was compared with the denatured protein formed is shown in Table IX. The rate of inactivation of the phage is proportional to the concentration of chymo trypsin, indicating a true catalytic reaction. Slightly more protein was denatured than corresponded to the loss in activity so that the protein remaining was slightly more active than the original but the difference is hardly outside the experimental error.

*Sedimentation of Phage Activity and of the Protein in the Ultracentrifuge*—It was stated above that the preparation was found to be homogeneous when examined in the analytical centrifuge. In the absence of other evidence, however, this result cannot be considered

proof of purity since Svedberg (20) has shown that proteins exist in groups having the same molecular weight, hence several proteins might be present and still present a solution homogeneous in regard to size

TABLE IX

*Inactivation of Phage by Chymo-Trypsin and Formation of Denatured Protein*  
 Phage solution in { 0.1 M pH 7.6 phosphate buffer 20°C  
 1/4 saturated ammonium sulfate

1 ml of this solution added to 5 ml N/1 acetic acid and protein determined by turbidity Samples centrifuged as noted and phage activity and protein nitrogen determined on suspension and on supernatant  
 Protein nitrogen 1 ml plus 5 ml N/1 acetic acid containing 0.01 mg pepsin nitrogen per ml 2 hrs at 30°C \*

Mg chymo- trypsin nitro- gen/ml	0				0.01				0.03				0.10			
	Suspension		Super-natant		Suspension		Super-natant		Suspension		Super-natant		Suspension		Super-natant	
	ph u	Protein N	ph u	Protein N	ph u	Protein N	ph u	Protein N	ph u	Protein N	ph u	Protein N	ph u	Protein N	ph u	Protein N
0	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10
1	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10	400	0.10
2																
4	400	0.10			100	0.10	100	0.01	100	0.10	100	0.05				

Loss in activity and protein nitrogen after 1 day 20°C with varying quantity chymo-trypsin				
Mg chymo-trypsin/ml..	0	0.01	0.03	0.10
[ph u] ml remaining	400	200	150	30
[protein N] ml remaining	0.10	0.03	0.026	0.007
[ph u] mg protein N remaining	4000	6600	6000	4300
[ph u] ml lost		200	250	370
[protein N] ml lost		0.07	0.074	0.093
[ph. u] mg protein N lost		2800	3400	4000

\* This process digests the chymo-trypsin which would otherwise interfere with the determination of phage protein

It is also known that some proteins may be denatured without change in molecular weight so that the method would fail to distinguish between native and denatured protein There is the further pos-

sibility that the active agent is present in too small concentration to detect or that it did not absorb ultraviolet light strongly. A much more sensitive test for the relation of protein to activity consists in centrifuging the solution in bulk and analyzing various layers of the centrifuged solution for activity and protein. The result of such an experiment is shown in Table X. The figures show that the relation of protein to activity is the same throughout the solution. Hence, the protein and the active agent sediment at the same rate. There was no loss of activity during this experiment but if the precipitate

TABLE X

*Ultracentrifuge*

6.5 ml phage 96-32 M/10 pH 7.6  $\text{PO}_4$  1 M  $(\text{NH}_4)_2\text{SO}_4$

Centrifuge  $3\frac{1}{2}$  hrs 0-10°C R P M—25 000 Wyckoff centrifuge

Solution removed in layers and analyzed for protein nitrogen and phage activity

Layer No	Vol.	[ph. u.] ml	[Protein N] ml	[ph. u.] mg protein N
Original solution	6.5	100	0.010	10.000
Top	1.0	0.007	<0.0001	
2	1.0	{ 3	0.0003	10.000
		{ 5		15.000
3	1.5	2.5	0.0003	8.000
		5.0		13.000
Sediment dissolved in $\text{PO}_4$ pH 7.6	2.0	200	0.026	8.000
		300		12.000

is resuspended and again centrifuged there is considerable loss in activity so that this method cannot be used as a means of purification. The inactive protein formed in this way has approximately the same sedimentation constant as the active protein (Wyckoff (22)).

*Absorption of Phage Activity and Protein by Bacteria*—Susceptible living or dead bacteria remove phage activity from solution. With living bacteria the phage is distributed between the bacteria and the solution in accordance with the normal distribution law while dead bacteria bind the phage irreversibly in accordance with the adsorption theory (Krueger (27)). This reaction is specific since non susceptible

bacteria do not exhibit this property and furnishes a sensitive test for the relation of the phage activity and the protein. The results of an experiment in which this relation is tested are shown in Table XI. Two phage preparations were used, one (60-16) contained some inert protein while the second (94-25) contained less. The bacteria removed relatively more activity than protein from the impure preparation so that the solution left showed a lower specific activity than the original. With the more active preparation the loss in activity was proportional to the protein removed. The bacteria therefore appear to take up only the active protein. Non-susceptible staphylococci or *coli* do not remove either protein or activity.

This property of removing phage from solution appears to furnish a very efficient method of purification but no practical method could be found to remove the phage from the bacteria although the phage may be removed by repeated washing. The solution obtained in this way, however, is too dilute to use as a means of preparation.

*Ultraviolet Absorption Spectra*—The ultraviolet absorption spectrum may be determined by direct measurement and the inactivating efficiency of the various wave lengths may be determined by inactivation experiments with monochromatic light. The relative absorption spectra may be calculated from such inactivation experiments, as Warburg has shown. If it is found that the relative absorption spectra for various wave lengths, as determined from inactivation experiments, agree with those found by direct observation very strong proof of the identity of the protein and the active agent would be obtained. The necessary apparatus for such inactivation experiments was not available but such measurements have been made by Gates (28) with a closely related bacteriophage. The relative absorption spectra calculated from Gates' measurements and that found with a purified phage preparation are shown in Fig 2. The two curves coincide very well from 2600-2900 Å  $\mu$  but below 2600 the curves diverge. The direct measurement on purified phage shows greater absorption in this region than indicated by Gates' inactivation experiments. This discrepancy may be due to the fact that Gates' measurements were carried out with a different phage or to the presence of some inert material in the present phage preparation which absorbs strongly in this region. It is also possible that light may be

TABLE XI

*Adsorption of Phage Protein and Phage Activity by Bacteria*

Bacteria grown on Blake bottles washed off, and centrifuged, washed with distilled water until supernatant protein free. Noted quantity of bacteria added to phage solution in  $\frac{1}{4}$  saturated ammonium sulfate, pH 7.4. Stood 0°C as noted centrifuged and phage activity and protein nitrogen determined in supernatant.

Bacteria	Living staphylococci (homologous)			Heat killed staphylococci (homologous)												Dead hemolytic staphylo cocc (heterologous)		Dead B cells	
	60-16			60-16						94-25						60-81	60-81	60-81	60-81
	Phage No.																		
Bacteria/ml mm <sup>3</sup>	0	2	4	10	0	0 02	0 04	0 1	0 2	0	0 1	0 2	1 0	1 0	1 0	1 0	1 0	0	0
Hours 0 C	3	3	3	3	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
[ph u] ml super																			
nant	60	25	10	4	70	30	20	5	2	250	165	100	18	18	18	25	20	20	20
[Protein N] ml su																			
permatant	0 018	0 012	0 006	0 002	0 017	0 016	0 010	0 008	0 003	0 03	0 02	0 015	0 003	0 003	0 003	0 0032	0 0031	0 0032	0 0031
[ph u] mg protein																			
N remaining	3300	2000	1600	2000	4000	1800	2000	600	700	8300	8250	6700	6000	6000	6000	8000	7000	8000	7000
[ph u] mg protein																			
N removed		6000	4500	3500			7000	7000	4200		8500	10 000							

absorbed in this region without causing inactivation. The phage preparation used showed a specific activity of about 5000 [ph u]/mg N and therefore undoubtedly contained quite a large amount of inactive protein.

A similar discrepancy has been found in the case of urease (28a) and also with pepsin (28b).

The extinction coefficient  $\left(\log_{10} \frac{I_0}{I} \text{ 1 cm cell}\right)$  of this solution at

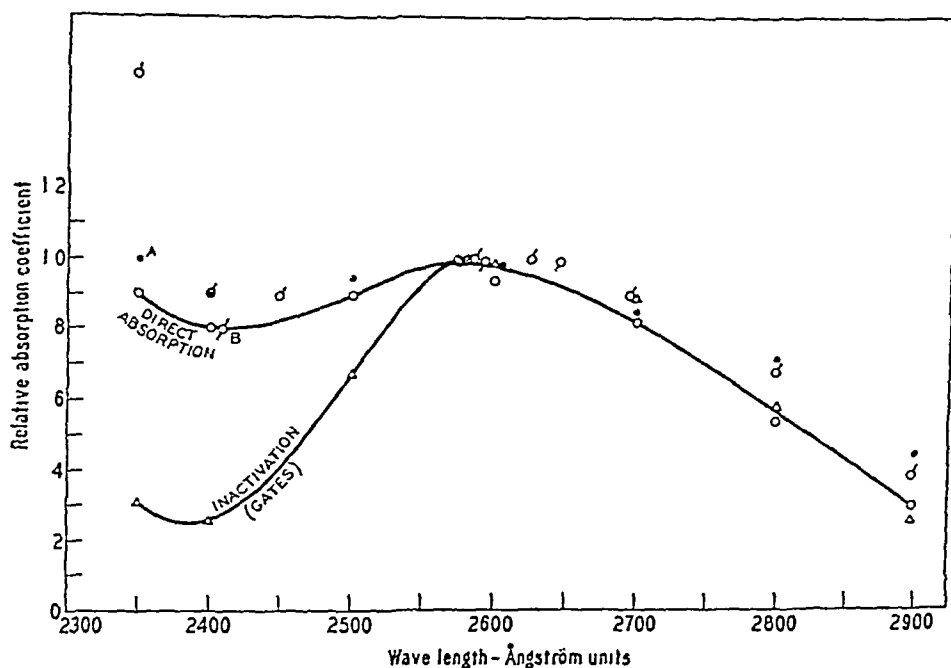


FIG. 2. Comparison of relative absorption of various wave lengths of ultra-violet light, as determined by direct measurement and by inactivating efficiency.

2600 Å u was 2 for a concentration of 0.1 mg protein/ml. The absorption varies with the concentration so that this figure cannot be corrected to unit concentration. This value is greater than that of most simple proteins and the shape of the curve is not that usually found for simple proteins.

The ultraviolet measurements were made by Dr. George I. Lavin.

*Solubility Determinations*—The preceding experiments show that the protein and activity are certainly very closely related and that the activity appears to be a constant property of the protein. Ex-

perience has shown, however, that a protein may behave in many respects as a pure chemical compound and yet, when tested by the solubility method, prove to be a mixture or solid solution of several different, although probably closely related, proteins. There is some indication in the experiments reported above that the preparation contains some inert protein, since the specific activity varies between

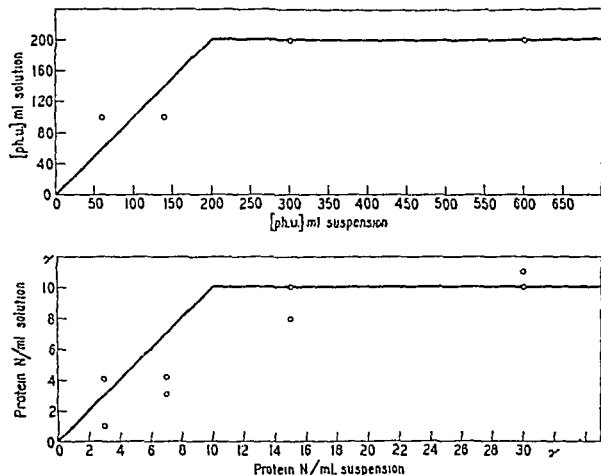


FIG. 3 Solubility of phage protein (specific activity 20,000 [ph u] mg N) in { 0.53 saturated ammonium sulfate in the presence of increasing quantity of 0.02 M sodium phosphate solid phage

5,000 and 10,000 [ph u] mg N and since it was not possible to obtain quantitative separation of denatured and native protein. Solubility determinations were therefore made and it was found that the solubility increased rapidly as the total concentration increased, showing that the solid phase consisted of more than one component. It was further found that the specific activity of the solution was slightly higher than that of the precipitate. These results indicated a further

method of purification In the earlier work the protein was precipitated from dilute solution with 0.5 saturated ammonium sulfate It appeared from the results of the solubility experiment that in more concentrated solution a large amount of activity would remain in solution in this concentration of ammonium sulfate and that this fraction would be more active Accordingly, many attempts were made to prepare a concentrated solution which could then be precipitated with 0.5 saturated ammonium sulfate The difficulty lies in the fact that at each precipitation more or less denatured protein is formed which can only be removed by redissolving in water, precipitating at 0.45 ammonium sulfate to remove the inert protein, and filtering The precipitate formed under these conditions carries with it more or less of the active protein so that again a dilute solution is obtained However, two preparations were obtained which had a specific activity of 20,000 [ph u]/mg nitrogen which is 2-3 times that of the material used in the earlier experiments This material was soluble to the extent of at least 0.10 mg/ml in 0.45 saturated ammonium sulfate and its solubility in 0.53 saturated ammonium sulfate varied only slightly with the total concentration This is a very rigorous test of purity and the results show that this preparation consisted largely of one component

#### DISCUSSION

The present work has shown that a substance with the general properties of a nucleoprotein may be isolated from lysed cultures and that the bacteriophage activity is very closely associated with the protein The bacteriophage, in common with the viruses, possesses the remarkable property of increasing when in the presence of the proper living cells In the case of bacteriophage, at least, the increase under certain conditions is autocatalytic, *i.e.*, it is proportional at any time to the amount already present This increase is analogous in many respects to the growth of cultures of unicellular organisms and, largely for this reason, bacteriophage and other viruses are assumed by many workers to be living organisms This assumption tacitly involves the further assumption of organized cellular structure and of metabolism for which, at present, there appears to be no evidence There is, indeed, no proof that the phage increases by



itself since the increase occurs only in the presence of living cells and since under these conditions, the normal proteins and enzymes of the cells are also increasing. In fact, it has been recently found by Kunitz (29) that an extracellular proteolytic enzyme produced by a mold (*Penicillium*) increases in an analogous manner. The only difference between the phage and "normal" enzymes or proteins, then, is the fact that not all cultures contain it. If all cultures did contain it, it would simply be regarded as a normal enzyme and the culture would be said to autolyze readily. The same is true of many viruses. Their characteristic property is not that they increase in the presence of living cells, since many other substances also increase, but that some cells, which previously did not produce any phage or virus, will do so after being inoculated with the active agent. It is possible that this property may be quite general and that many substances would be found to increase when added to living cells if it were possible to first obtain cells which did not already have some of the substance present. A case in point is the formation of the specific pneumococcus polysaccharide. Griffith (30) found that under certain conditions pneumococci could be freed from their capsules and grown without forming any specific capsular material. If capsular material from a different type is now added the culture produces indefinite quantities of this latter type. For example, a Type II culture grown under conditions which result in loss of Type II capsule may be inoculated with Type III capsular material which then increases in the culture. The production of antibodies may be a related phenomenon, although in this case the antigen itself does not increase but instead a new protein, the antibody, increases.

Gratia (38) has shown that thrombin increases during the clotting of blood and has pointed out the analogy between this reaction and the production of phage. The mechanism assumed for the formation of bacteriophage outlined in the following is similar to that proposed by Bordet (6).

Two cases in which such autocatalytic production of a protein may be obtained *in vitro* have recently been studied in detail. An inert protein which does not differ in any striking way from many other proteins was isolated from pancreas. If a solution of this inert protein is inoculated with a trace of active trypsin all the inert protein